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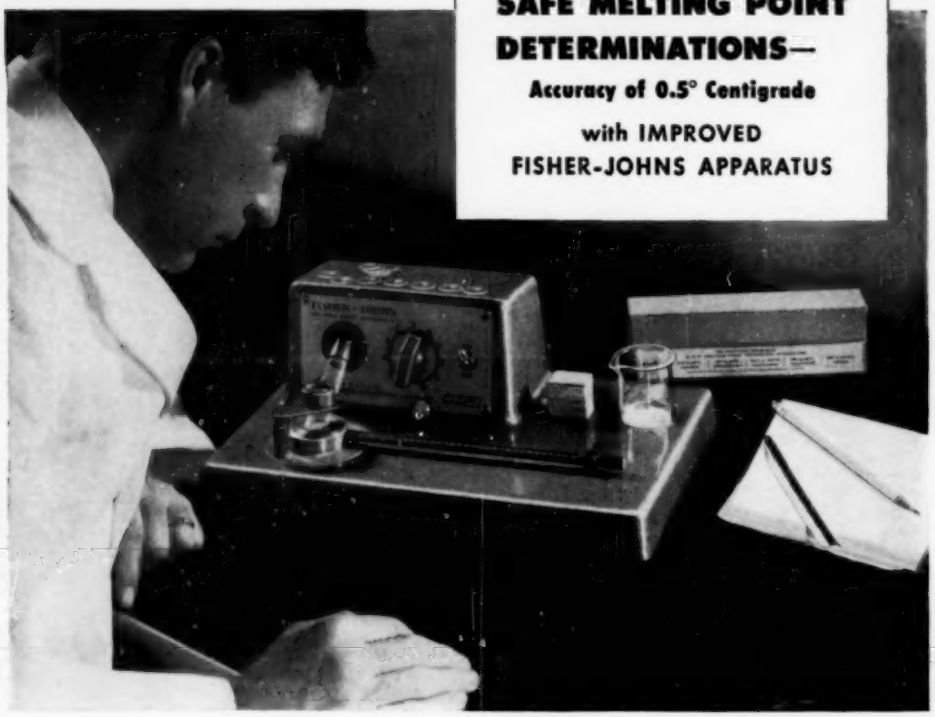

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NOTICE

PAPERS FOR SECTION MEETINGS OF THE AMERICAN PHARMACEUTICAL ASSOCIATION

The next annual meeting of the American Pharmaceutical Association will be held in Miami Beach, Florida, May 1 to 7, 1955. Members planning to present papers before any section during this meeting are earnestly requested and strongly urged to notify the appropriate Section secretary of their intention promptly. Titles and abstracts (not to exceed 200 words) of papers should be submitted by February 1, 1955, or preferably earlier, in order to insure a place on any section program.

The Journal of the American Pharmaceutical Association is published monthly by the American Pharmaceutical Association in two editions: Scientific Edition and Practical Pharmacy Edition, under the supervision of the Committee on Publications of the Council of the Association. Publication office: 20th and Northampton Streets, Easton, Pennsylvania.

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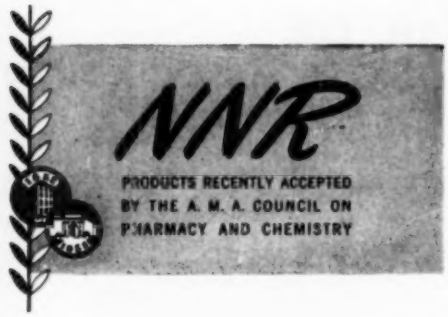

PROTOVERATRINES A and B.—Veralba (Pitman-Moore).—Protoveratrines A and B is a mixture of two alkaloids, isolated by appropriate means from *Veratrum album*. The structural formula of the two alkaloids is not known.

Actions and Uses.—Protoveratrines A and B exert their primary effect on the cardiovascular system by their influence on buffer-reflex receptors; with therapeutic doses the mixture induces vasodilation through effects at those sites. Some investigators believe that this results in a normal physiological redistribution of blood to all vascular beds, resulting in postural hypotension that is less severe and less frequent than with ganglionic blocking agents. Comparison of the two components of protoveratrine used in experimental animals reveals no qualitative differences in action, but protoveratrine B has about 80% of the potency of protoveratrine A.

Protoveratrines A and B may be useful in the symptomatic treatment of essential hypertension, acute or chronic renal hypertension, malignant hypertension, hypertension associated with toxemia of pregnancy, and carcinoma of the adrenal cortex. Adequate dosage of the mixture produces a significant decrease in the diastolic and systolic blood pressures in most patients. This decrease is much more certain after intravenous or intramuscular injection than after oral administration. As with other Veratrum alkaloids, response of different patients varies considerably, and, by the oral route, response of an individual patient occasionally varies.

Because of their powerful hypotensive effect, parenteral injection of protoveratrines A and B should be reserved chiefly for the management of hypertensive crises in which prompt lowering of the blood pressure and readily controlled dosage are essential. The oral route is suitable for the management of mild to severe hypertension in some patients. When pronounced impairment of renal function exists, adequate control of the hypertension is unlikely. Because of their hypotensive action, protoveratrines A and B may alleviate such symptoms as headache, insomnia, delirium, dizziness, blurred vision, and nervousness. Their slowing effect on the heart rate may be followed by a reduction in the degree of congestive heart failure when this is caused by left ventricular failure associated with hypertension. Protoveratrines A and B also reduce hypertensive pulmonary edema and lower the elevated blood pressure occasionally encountered with cortisone therapy; they may also be useful in controlling convulsions of eclampsia.

As with other Veratrum alkaloids, overdosage of protoveratrines A and B produces disturbing, toxic side-effects, and with therapeutic dosage their vasodilator action is regularly accompanied by some cardiac slowing. In approximate order of appearance, side-reactions include feeling of warmth or flushing, bradycardia, nausea, salivation, vomiting, cardiac arrhythmia, excessive hypotension, and circulatory collapse. Occasionally a feeling of substernal pain or tightness is experienced. Unless bradycardia is severe and associated with arrhythmias, it is not necessarily harmful and may be desirable in cases of tachycardia with circulatory failure. Severe bradycardia may be overcome by an intravenous or intramuscular injection of 0.4 mg. of atropine sulfate. Parenteral injection, especially when administered too rapidly by the intravenous route, may produce sudden, excessive hypotension accompanied by collapse. This can best be treated by intramuscular injection of vasopressor drugs such as ephedrine (25 mg.) or phenylephrine (5 mg.). A feeling of warmth over the epigastrium, perineum, face, or extremities is commonly observed, but this reaction is of minor importance and usually not unpleasant; however, gross irregularity of the pulse and nausea or vomiting appearing during intravenous administration indicate the beginning of overdosage and the need to discontinue injection. These signs (particularly nausea that is not previously present) serve as a guide to the tolerated dosage. Protoveratrines A and B should be employed cautiously in chronic uremia because such patients may have difficulty in adjusting to lowered blood pressure levels. Caution is also necessary in the presence of digitalis intoxication. Protoveratrines A and B are contraindicated in hypotension and high intracranial pressure not secondary to hypertension.

Dosage.—Protoveratrines A and B are administered orally, intravenously, or intramuscularly. Since the effective therapeutic dose is sometimes close to the dose that produces undesirable side-reactions, it is essential to establish carefully each patient's optimum dosage schedule. Usually this can be done best if the patient is hospitalized. The stabilized resting diastolic and systolic blood pressures should be determined prior to initiating therapy.

For the management of moderate hypertension the usual starting oral dose for adults is 0.5 mg. after each meal and at bedtime. The blood pressure should be determined two to three hours after each dose if the patient is hospitalized. In the ambulatory patient a daily recording of the blood pressure should be made two to three hours after the noon or evening dose. If the blood pressure is not significantly lowered, each of the four doses may be increased 0.2 mg. for the next day. Subsequent daily doses may be increased similarly until a satisfactory response is obtained. If nausea, vomiting, or other side-effects appear before an effective dosage level is established, the dose should be reduced by 0.1 or 0.2 mg., as may be necessary to obtain the desired effect just short of the signs of overdosage. The average effective dose varies from 0.4 to 1.5 mg. four times daily. Shorter or longer intervals may be used; or differential doses, such as a larger morning

or bedtime dose with smaller interim doses, may be more effective in some patients.

Parenteral injection for the management of hypertensive crises should be initiated by the intravenous route according to one of the following methods:

1. An initial dose of 0.06 to 0.1 mg. is slowly administered. If no significant decrease in blood pressure occurs, an increment of 0.02 mg. may be repeated in four hours; and, if necessary, the dose may be increased by the same increment at four hour intervals until the desired response is obtained. As the optimal response is approached, increments of 0.01 mg. are preferable. When toxic signs occur, one or two doses may be omitted and therapy recommenced at a lower dose. If a particularly prompt effect is necessary, the initial dose may be followed by small doses of 0.02 mg. at 15 minute intervals. Maximum response usually appears 10 to 30 minutes after intravenous injection. Duration of action of a single intravenous dose extends about one and one-half to three hours, but cumulative effects may result even when injections are spaced at longer intervals.

2. Slow intravenous infusion may be employed by using a more dilute solution prepared by dissolving 2 mg. of protoveratrines A and B in 200 cc. of either isotonic sodium chloride solution or 5% dextrose to make a concentration of 0.001 mg. per cc. Infusion of this dilution at the rate of 3 to 6 cc. every ten minutes will usually decrease blood pressure significantly, and 1 to 3 cc. administered each 10 minutes is the approximate maintenance rate.

3. An alternate method of interrupted injection is use of a 10 cc. syringe dilution of 0.1 mg. in either isotonic sodium chloride solution or 5% dextrose to make a concentration of 0.01 mg. per cc. This dilution is given at the rate of 0.5 cc. per minute for eight minutes (total 4 cc.), during which time the blood pressure is continuously observed. After an interval of two minutes the same rate is continued for six more minutes (3 cc.; total 7 cc.). After another two-minute interval the injection is continued at the same rate for an additional six minutes, during which time the blood pressure is checked closely (total 10 cc., which exhausts the supply in the syringe). The injection should be interrupted whenever either the systolic or diastolic pressure falls 20 mm. Hg. Three minutes is allowed for stabilization of blood pressure at the new level. If no fall results from the first 10 cc., five minutes should elapse; then the syringe is refilled with the same dilution, and the previous procedure is repeated. The amount required may range from 5 to 20 cc. or more, but the average effective dose is 8 to 16 cc. (0.08 to 0.16 mg.).

After the stabilization of initial response the effect may be maintained by slow intravenous infusion as described under paragraph 2. The patient should be under constant observation, and the blood pressure should be checked at least every 10 to 15 minutes. Care should be taken to avoid a period of rapid infusion while the rates of flow are adjusted. Some clinicians may prefer to use the dilute syringe method instead of continuous infusion to maintain the effect of the initial injection, repeating that procedure after the blood pressure has returned to a hypertensive level.

Intramuscular injection can also be used to maintain the initial response to intravenous therapy. The mixture is administered in doses of 0.16 to 0.4 mg. every four to eight hours. An alternative method is to inject an initial dose of 0.12 mg., taking the blood pressure every fifteen minutes thereafter. The maximum effect usually appears within one to two hours. If the desired response does not occur, a dose of 0.16 mg. may be repeated after an interval of not less than four hours. This may be followed with 0.04 mg. increments not oftener than every four hours until desired lowering of the blood pressure results. The dose established by this method usually may be repeated if the interval between injections is not less than four hours. Six or eight hour intervals may also be effective.

Tests and Standards.—

Physical Properties: Protoveratrines A and B is a white, odorless, slightly bitter, crystalline powder with a strongly sternutatory action, m.p. 256–262° (with decomposition). It is freely soluble in chloroform, very slightly soluble in ether, and practically insoluble in petroleum ether and in water. Protoveratrines A and B is stable to light and air. It is stable for several months in solutions of *p*H 4.0–6.0, but is rapidly destroyed in basic and alcoholic solutions. The *p*H of a saturated solution is 6.5–7.3. (The *p*H of the 0.02% solution, containing 0.2% *m*-cresol, is 5.6–6.2.)

Purity Tests: Suspend about 5 mg. of protoveratrines A and B in 5 ml. of sulfuric acid: no color is produced within one minute (*absence of certain other veratrum alkaloids and alkamines*): a pink color, which exhibits a blue-green fluorescence under ultraviolet light, appears in two to three minutes and gradually deepens in intensity.

[For more information regarding this monograph, see *J. Am. Med. Assoc.*, **156** 1250 (November 27, 1954).]

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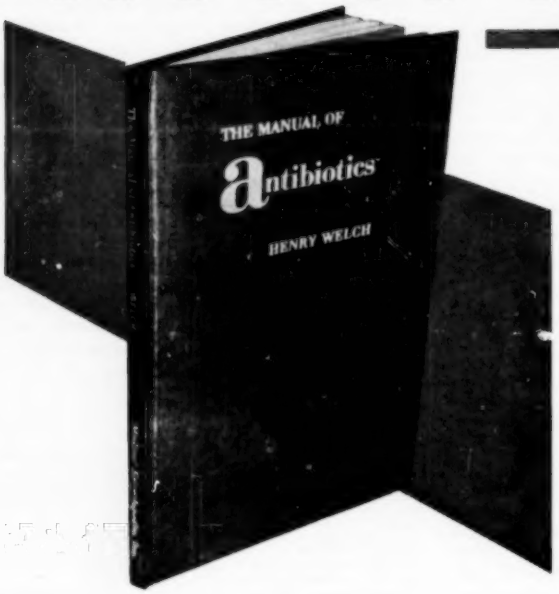

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Nominations are now being received by the AMERICAN PHARMACEUTICAL ASSOCIATION for the 1955 Chilean Iodine Educational Bureau, Inc. Award recognizing outstanding research in the chemistry and pharmacy of iodine and its compounds as applied in pharmacy or medicine.

The award consists of $1,000 and a diploma setting forth the reasons for selection of the recipient. It may be presented each year at the annual meeting of the ASSOCIATION.

Any member of the ASSOCIATION may propose a nominee by submitting eight copies of each of the publications to be considered in the competition, a biographical sketch of the nominee including date of birth, and a list of his publications. Eight copies of the nomination must be submitted to Robert P. Fischelis, Secretary of the AMERICAN PHARMACEUTICAL ASSOCIATION, 2215 Constitution Ave., N. W., Washington 7, D. C. To be eligible for the 1955 Award, *nominations must be received on or before January 1, 1955*.

A nominee must be a resident of the United States or Canada. He must have accomplished outstanding research in the chemistry or pharmacy of iodine and its compounds as applied in pharmacy or medicine.

During the period covered by the nomination the nominee shall have been actively engaged in, shall have completed, or shall have published a report upon the line of investigation for which the award is made. During the period of two years prior to the date of nomination, the nominee shall not have been engaged in research under the sponsorship of the Chilean Iodine Educational Bureau, Inc.

The recipient will be selected by an award committee which is appointed by the chairman of the ASSOCIATION's Council. The present committee includes Justin L. Powers, chairman; Frederick F. Blicke, Paul Block, E. A. Brecht, Leroy D. Edwards, Lloyd M. Parks, and Stanley G. Mittelstaedt.

The recipient will deliver a paper or lecture upon the subject of his scientific work at the meeting at which the award is conferred. His paper, or address, will then be published in the JOURNAL OF THE AMERICAN PHARMACEUTICAL ASSOCIATION. Travel expense to the meeting at which the award is to be made is provided for up to $250.

The award is now in its seventh year. The recipient of the first award was Dr. William T. Salter, Yale University pharmacologist; the second recipient was Dr. George M. Curtis, Chairman of the Department of Surgical Research and Professor of Surgery at Ohio State University, and the third was Dr. C. P. Leblond, Professor of Anatomy, McGill University, Montreal Canada. The fourth award was shared jointly by Dr. George Moore of the University of Minnesota Medical School and Dr. Moses Ashkenazy, Chief of Neurosurgery, Veterans Hospital, Houston, Tex. Dr. Vernon H. Wallingford, Mallinckrodt Chemical Works, St. Louis, Missouri, was the recipient of the fifth award. Dr. Jack Gross, Associate Professor of Anatomy, State University of New York, College of Medicine, in Brooklyn received the sixth award which was conferred during the 1954 Convention of the AMERICAN PHARMACEUTICAL ASSOCIATION.

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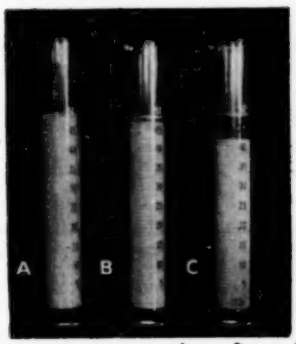

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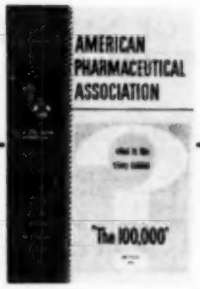

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In the December 1954 issue of the Practical Pharmacy Edition

(All references to actions and uses are taken from the statements of the producers of the products mentioned.)

▸ **Abbott Laboratories** has used the second cover and four additional pages for *multi-vitamin* **Optilet** tables, *iron-plus* **Iberol,** and *filmtab* **Erythrocin Stearate,** an enteric coated, oral antibiotic.

▸ Its *integrity* is the trait emphasized by **The American Druggists' Fire Insurance Co.,** "The Druggists' Own Insurance Co."

▸ **Decholin, Decholin Sodium** and **Decholin** *with* **Belladonna** are advised by the **Ames Company** for use in the treatment of hepatobiliary disorders.

▸ "First aid for throat irritations," is the descriptive phrase **Hynson, Westcott & Dunning** applies to **Thantis Lozenges.**

▸ In *Phil's Pharmacy,* **Lederle Laboratories** graphically tells the sales story of **Achromycin,** described as "'the original brand" of tetracycline.

▸ Recommended for a nutritional *lift* is **Lilly's 'Becotin with Vitamin C'.**

▸ **Adrenosem, Aminodrox, Broniacin, Livitamin, Massengill Powder, Obedrin, Salcedrox** and **Semhyten** are the prescription specialties catalogued by the **S. E. Massengill Company** in its insertion.

▸ **Merck & Co.** in their announcement feature their familiar seal as a well known "symbol of professional trust."

▸ **Chloromycetin** (chloramphenicol, **Parke-Davis**) is an antibiotic used in the treatment of "more than eleven million patients to date."

▸ "Today's apprentice, tomorrow's pharmacist" is the future assured the conscientious student enrolled in the **Philadelphia College of Pharmacy and Science.**

▸ **Roetinic** is described by Roerig as "the only one-a-day hematinic which contains therapeutic quantities of all known hemapoietic factors."

▸ **Tracinets, Sharpe & Dohme** antibiotic trochés, are highlighted as supplementing systemic therapy in severe throat infections.

▸ Indorsement of **Benzedrex Inhalers'** rapid turnover is the theme of one of the Smith, Kline & French advertisements; in another, S.K.F. announces its newest 'Spansule' capsules, **Prydon** and **Prydonnal;** and in still another, emphasizes the wide diversity of **Thorazine's** activity.

▸ In two insertions, **Upjohn** discloses that diplicoccus pneumoniae and staphylococcus aureus are but two of "more than 30 organisms susceptible to **Panmycin.**"

▸ The **Warner-Chilcott** announcement introduces new sizes and forms of **Peritrate** (prophylaxis against angina pectoris) and **Methium** (hypotensive agent).

▸ In what it describes as "an alliance of the classic and contemporary," **Winthrop-Stearns** focuses attention on *new* **Theominal R. S.** "for essential hypertension."

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Pharmacological Properties of Mephentermine, a Sympathomimetic Amine*

By DONALD K. ECKFELD, LIESE L. ABELL,† and JOSEPH SEIFTER

Mephentermine, N-methyl-ω-phenyl-tertiary-butylamine, was compared with epinephrine, amphetamine, *d*-desoxyephedrine and *l*-ephedrine. Although unique in lacking an asymmetric carbon atom and therefore not having a *d*- or *l*- form, its sympathomimetic properties are similar to those of the other β-phenylethylamines. It is less toxic to mice and causes less cerebral stimulation in chicks, cats, and dogs than amphetamine or *d*-desoxyephedrine. The pressor response and effect on heart rate in intact and vagotomized dogs are qualitatively and quantitatively similar to those of *l*-ephedrine. Like *l*-ephedrine it constricts the small vessels of the nasal mucosa and increases the air space of the nasal passage. Absorption following sinus instillation is minimal and is no greater than *l*-ephedrine. Unlike epinephrine it does not cause hyperglycemia in rabbits. It causes pupillary dilatation, is not anesthetic when applied topically to the cornea, is weakly spasmolytic for smooth muscle of intestine, and has no effect on tracheal cilia.

SINCE THE preliminary report by Seifter, *et al.* (1), wherein it was first demonstrated that mephentermine[1] had sympathomimetic properties similar to those of other β-phenylethylamines, a number of investigators have directed their attention toward characterizing its cardiac effects both in animals (2–4) and in patients suffering from shock accompanying myocardial infarction (5–8). The purpose of this paper is to present additional laboratory data to define further the pharmacological properties of this agent and to compare its actions with those of amphetamine sulfate, *d*-desoxyephedrine hydrochloride, and *l*-ephedrine hydrochloride.

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Presented, in part, at the meeting of the American Chemical Society, Atlantic City, N. J., September 18–23, 1949.
† Present address: Goldwater Memorial Hospital, Welfare Island, N. Y.
[1] Wyamine® Wyeth Laboratories, Inc.: N-methyl-ω-phenyl-tertiary-butylamine.

EXPERIMENTAL

Chemical and Physical Properties.—Mephentermine chemically resembles many of the commonly used sympathomimetic amines. It differs, however, in that the amino group is attached to a tertiary carbon atom and since it contains no asymmetric carbon atom it has no *d*- and *l*- forms (Table I). The base boils at 82–83°/7 mm., has a refractive index of n_D^{20} 1.5110, a density of d_{20}^{20} 0.9231, and the pK_A value at 30° is 10.35. At 22.5° its volatility is 1.26 mg./L. of dry air. The sulfate crystallizes as a dihydrate and melts with decomposition at 215–217°. The *p*H of 1% solutions of the anhydrous sulfate in distilled water at 20° is 5.6.

Acute Toxicity.—The LD_{50}'s determined in albino mice weighing 16–25 Gm. are summarized in Table I. Each value was computed according to the method of Bliss (9) using five or more logarithmically spaced doses. The animals were observed for seven days and deaths occurring within this period were included in the calculations. After intra-abdominal administration mephentermine was approximately

TABLE I.—SUMMARY OF ACUTE INTRA-ABDOMINAL TOXICITY

	Chem. Structure						No. Mice Used	Quant. Estn. of LD_{50} (mg. Base/Kg.)	Range (±2 S. E.) Limits Lower (mg. Base/Kg.)	Upper	Slope (Dosage Mort. Curve) b	Relative Toxicity
Mephentermine Sulfate	H	H	H	CH_3	CH_3	CH_2	60	89.4	84.3–	94.7	17.6	1.0
Amphetamine Sulfate	H	H	H	CH_2	H	H	80	59.7	47.5–	75.1	3.7	1.5
l-Ephedrine Hydrochloride	H	H	OH	CH_3	H	CH_2	50	276.9	255.0–	300.8	15.0	0.3
d-Desoxyephedrine Hydrochloride	H	H	H	CH_3	H	CH_2	50	63.1	56.8–	70.2	10.3	1.4

TABLE II.—EFFECT ON BLOOD GLUCOSE LEVELS OF RABBITS

Comp. Admn.	Dose, (mg. Base/Kg.)	Route Admn.	Mg. % Blood Glucose (Min. after Injection) Pretreat.	5	10	15	30	45	60	90	120
Epinephrine	0.25	Subcutaneous	135	...	...	170	204	...	258	...	...
	1.0	Subcutaneous	110	...	...	100	90	...	118	...	...
	10.0	Subcutaneous	102	100	103	115	102	104	103	102	102
	10.0	Subcutaneous	99	95	95	95	105	105	99	...	...
Mephentermine	20.0	Subcutaneous	105	105	112	...	100	80	108	102	...
	20.0	Subcutaneous	105	115	108	...	110	120	120	118	135
	1.0	Intravenous	99	96	98	105	105	104	98	105	99
	2.0	Intravenous	100	99	99	108	106	99	99	99	99

TABLE III.—EFFECTS OF MEPHENTERMINE, EPINEPHRINE, AND *l*-EPHEDRINE ON BLOOD PRESSURE, HEART RATE, AND NASAL VOLUME OF DOGS

Dog No.	Vagi	Comp. Admn.	Dose (mg. Base/Kg.)	Pretreat. Blood Pressure[a]	Pretreat. Heart Rate[b]	After Drug Injection Blood Pressure[a]	After Drug Injection Heart Rate[b]	Time to Return to Pretreat. Level (Min.)	Increase in Nasal Volume Duration (mm.)	Increase in Nasal Volume Duration (Min.)
1		Epinephrine	0.003	184	...	260	...	3	12	3.5
	Intact	Mephentermine	0.4	187	132	240	122	8	15	10.0
		Ephedrine	0.4	194	134	250	118	8	15	10.0
2		Epinephrine	0.003	140	116	266	130	3	23	3.5
	Severed	Mephentermine	0.3	144	112	190	110	11	20	10.5
		Ephedrine	0.3	162	112	210	110	9.5	19	11.0
3		Epinephrine	0.006	168	108	232	120	3	20	3.5
	Severed	Mephentermine	0.5	170	110	214	130	6.5	19	10.0
		Ephedrine	0.5	180	122	222	102	8	20	11.5
4		Epinephrine	0.003	210	116	270	124	3	17	2.5
	Severed	Mephentermine	0.5	232	128	268	120	11	16	11.0
		Ephedrine	0.5	204	118	258	120	10	13	8.5
5		Epinephrine	0.004	178	100	288	132	2.5	16	3.5
	Severed	Mephentermine	0.6	174	100	260	120	14	15	15.0
		Ephedrine	0.6	194	100	250	126	12	9	10.0

[a] Mm. Hg.
[b] Beats per minute.

two-thirds as toxic as amphetamine or *d*-desoxyephedrine and three times more toxic than *l*-ephedrine. Symptoms of toxicity caused by mephentermine at the LD_{50} consisted of Straub tail, sensitivity to extraneous stimuli, and tremors. The other amines showed similar symptoms at their LD_{50}; however, in addition *l*-ephedrine caused depression for variable periods of time prior to the onset of tremors and *d*-desoxyephedrine and amphetamine caused salivation and convulsions. The tremors caused by amphetamine were more severe than those resulting from mephentermine. Deaths in all cases were due to respiratory arrest.

The approximate acute intravenous LD_{50} of mephentermine was determined in 10 rabbits. At 8 mg./Kg. the animals had dilated pupils, were apprehensive, breathed more rapidly than normally, and tremored; all survived. At 18 mg./Kg. similar symptoms were observed and all of the animals died within twenty-four hours. Thus the approximate LD_{50} appeared to be 13 mg./Kg.

Administration of sublethal doses of either mephentermine or epinephrine to groups of rabbits as shown in Table II demonstrated that, unlike epinephrine, mephentermine in doses of 1 to 20 mg./Kg. subcutaneously or 1 to 2 mg./Kg. intravenously did not cause hyperglycemia.

Effects on Blood Pressure, Heart Rate, and Nasal Volume.—Eighteen dogs were anesthetized with sodium pentobarbital and the carotid artery cannulated; blood pressure was recorded by means of a mercury manometer in groups of animals with and

without the vagi severed. Nasal volume was determined according to the technique described by Jackson (10). Table III shows that mephentermine closely resembles *l*-ephedrine qualitatively and quantitatively as to effect on blood pressure, heart rate, and ability to shrink nasal mucosa. Although mephentermine and *l*-ephedrine were approximately 100 times less potent than epinephrine, their duration of action at equipressor doses was three to five times longer. Unlike epinephrine, however, mephentermine caused tachyphylaxis wherein the dogs showed progressively decreasing pressor responses and even complete reversal after repeated injections. Dibenamine effectively blocked or reversed the characteristic pressor responses of both mephentermine and epinephrine. Mephentermine enhanced and prolonged the pressor responses of subsequently injected epinephrine and was effective against hexamethonium induced hypotension; the latter, however, was of short duration. That is, 3 mg./Kg. mephentermine elevated the pressure from approximately −36 mm. Hg to approximately +25 mm. Hg; this lasted three to five minutes and then gradually declined to the previous hypotensive level over twenty to forty minutes.

Absorption of mephentermine and *l*-ephedrine from the frontal sinuses was determined in 12 dogs by recording the changes in carotid artery blood pressure; mephentermine was instilled 23 times and *l*-ephedrine 21 times. In each case the amine was permitted to remain in contact with the sinus mucosa for five to one hundred and twenty minutes and then rinsed out prior to the next instillation. The pressor responses obtained following such contact, as shown in Table IV, indicate that mephentermine was not absorbed to any greater degree than *l*-ephedrine and that absorption was minimal for both amines. The apparent decrease in absorbability from the sinuses as manifested by depression of blood pressure (−9 to −12 mm. Hg) can be accounted for by tachyphylaxis due to repeated instillations.

TABLE IV.—EFFECTS OF ABSORPTION FROM FRONTAL SINUS ON DOG BLOOD PRESSURE

	Mephentermine	Ephedrine
No. instillations	23	21
Mg./Kg.	5–16 (av. 9)	5–16 (av. 9)
Duration of contact (min.)	5–120	5–64
No. contacts causing 10 or more mm. Hg rise	4	8
Av. rise, mm. Hg/group	+3.5	+6.5
Range, mm. Hg/group	−12 to +35	−9 to +16

TABLE V.—MINIMAL DOSES CAUSING CEREBRAL STIMULATION

	Chicks		Dogs		Cats	
Comp. Admn.	No.	mg./Kg.	No.	mg./Kg.	No.	mg./Kg.
Mephentermine Sulfate	80	39	10	20	8	25
Amphetamine Sulfate	50	13	9	8	10	7
d-Desoxyephedrine Hydrochloride	50	26	6	10	5	10

Cerebral Stimulation.—Mephentermine was compared with amphetamine and *d*-desoxyephedrine for cerebral stimulation in chicks, cats, and dogs (Table V).

Chicks were injected as described by Clymer and Seifter (11) and the minimal dose required to produce cerebral stimulation manifested by the twittering response was determined. By this technique mephentermine was less potent as a cerebral stimulant than either of the other two amines; *d*-desoxyephedrine and amphetamine were one and one-half and three times more effective, respectively.

Similarly, dogs and cats were less stimulated by mephentermine than by amphetamine and *d*-desoxyephedrine; the latter amines were more than twice as effective. Dogs exhibited characteristic symptoms twenty to forty minutes after subcutaneous injection of an effective dose of the amines which consisted of neck-craning with rapid side-to-side head movements; this was followed by panting, circus movements, dilatation of pupils, salivation, and lack of response to painful stimuli in some animals. These effects lasted several hours after which the animals were depressed. Cats showed dilated pupils, pilo-erection, salivation, unprovoked sham rage and hallucinatory and circus movements.

Barbiturate Potentiation.—Using the method described by Lamson, *et al.* (12), mephentermine was compared with epinephrine (Table VI) as a potentiator of thiopental and hexobarbital. Mephentermine, like epinephrine, potentiated thiopental anesthesia in mice given subanesthetic doses; however, the duration of the side position was shorter (one to three minutes) than that seen after epinephrine (five minutes) and the dose of mephentermine required to induce toleration of the side position was twice as great. Mephentermine in similar doses did not potentiate hexobarbital and, therefore, differed in this respect from epinephrine.

Effect on Smooth Muscle, Cornea, and Tracheal Cilia.—The effects of mephentermine and amphetamine were determined on strips of rabbit jejunum by the method of Magnus (13); Sollmann-Rademaekers solution containing 0.1% dextrose was used as the bath. Neither compound was a potent spasmolytic; however, the former was slightly more effective and induced slight to moderate relaxation at doses of 100 μg./ml. At this concentration both were ineffective in preventing or counteracting the spasmogenic action of physostigmine.

The local anesthetic activity of 1% solutions of mephentermine, amphetamine, and *d*-desoxyephedrine was investigated by instilling 0.5 ml. of each into the conjunctival sac of groups of six rabbits. None of the amines caused topical anesthesia and did not produce corneal pitting, redness, or congestion of the conjunctiva. All, however, caused pupillary dilatation which was comparable in degree and duration.

Immersion of strips of tracheal tissue from rabbits for fifteen minutes in 0.025 *M* (1.06%) mephentermine dissolved in Locke's solution, according to a modification of Proetz's technique (14, 15), produced no noticeable decrease in normal ciliary activity and no marked slowing was observed even after exposure for two hours. Similar results were obtained with amphetamine (0.92%) and *l*-ephedrine (1.07%).

TABLE VI.—POTENTIATION OF BARBITURATE ANESTHESIA IN MICE

Barbiturate Admn.	Dose (mg./Kg.)	Epinephrine (μg./Mouse)	Mephentermine (mg. Base/Kg.)	Total No. Mice	No. Anesthetized
Thiopental (Pentothal)	15	..	..	3	0
	10	..	..	3	0
	10	30	..	3	3
	5	30	..	3	0
	15	..	10	3	3
	15	..	5	3	3
	10	..	15	3	0[a]
Hexobarbital (Evipal)	50	..	..	3	3
	25	..	..	3	0
	25	30	..	3	3
	10	30	..	3	0[b]
	25	..	5	3	0
	25	..	10	3	0[a]
	25	..	15	3	0[a]

[a] Incoordination.
[b] Depression.

SUMMARY AND CONCLUSIONS

1. Mephentermine has sympathomimetic properties similar to that of other β-phenylethylamines. It is unique in that it contains no asymmetric carbon atom and therefore does not exist in *d*- or *l*- form.

2. It is less toxic for mice than amphetamine or *d*-desoxyephedrine and more toxic than *l*-ephedrine.

3. Mephentermine, unlike epinephrine, does not cause hyperglycemia in rabbits.

4. The pressor response and effect on heart rate due to mephentermine, in intact and vagotomized dogs, are qualitatively and quantitatively similar to those observed after *l*-ephedrine. Absorption from the frontal sinuses of dogs is minimal and the effect on blood pressure after such instillation is no greater than after *l*-ephedrine.

5. By constricting the small blood vessels of the nasal mucosa mephentermine is capable of increasing the air space of the nasal passages.

6. Mephentermine has approximately one-third to one-half the potency of amphetamine or *d*-desoxyephedrine as a cerebral stimulant for chicks, cats, and dogs.

7. It causes no decrease in normal ciliary activity of the trachea, lacks spasmolytic action on smooth muscle of the intestine, causes no anesthesia when applied topically to rabbit eyes, and, in common with other sympathomimetic amines, produces pupillary dilatation.

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Ultraviolet Spectrophotometric Determination of Reserpine*

By E. H. SAKAL and E. J. MERRILL

Methods are described for the determination of reserpine in crude extracts of Rauwolfia root and in fractions of varying degrees of purity. Relatively pure preparations are assayed directly by ultraviolet spectrophotometric methods comprising appropriate corrections for ultraviolet absorbing contamination. Cruder preparations are assayed for reserpine by ultraviolet spectrophotometry after a preliminary separation of the reserpine from the mixtures by paper ionophoresis. Methods are also described for the reserpine assay of tablets prepared either with crystalline reserpine or with mixtures of Rauwolfia alkaloids.

RESERPINE, a colorless, crystalline alkaloid (1–5) reported to possess hypotensive and other valuable pharmacological properties (1, 6, 7), occurs with a number of other alkaloids, in the roots of certain species of the botanical genus *Rauwolfia* L. of the *Apocynaceae*. The species which are known to contain reserpine are *R. serpentina* (1–4), *R. micrantha* (8), *R. canescens* (9), and *R. heterophylla* (5). This paper describes methods of assay of reserpine as the crude drug in Rauwolfia root extracts and fractions obtained therefrom, including the more refined fractions containing crystalline reserpine. There is also described a method of assay of reserpine in tablets and a characterization of the ultraviolet absorption spectrum of pure reserpine.

Reserpine possesses well-defined UV absorption characteristics which make possible a direct, quantitative evaluation of this substance by UV spectrophotometry. However, the presence of UV absorbing contaminants interferes with the evaluation although the effect of the contaminants may, in certain cases, be allowed for in the form of a calculated correction. In other cases the interference prevents a direct determination of reserpine by the UV method.

An analytical procedure has now been devised for the separation of reserpine from UV absorbing contaminants, particularly from other Rauwolfia alkaloids. The resolution is accomplished by the technique of paper ionophoresis whereby a number of the alkaloids migrate as cations in 5 *N* acetic acid-impregnated filter paper, under the action of an applied electrical field. The pattern of the resolved mixture is conveniently visualized by fluorescence of the zones induced by a UV lamp fitted with a 366-mμ filter. Such a pattern, so visualized is illustrated by the photograph in Fig. 4 in which reserpine appears as the slowest migrating alkaloid, characterized by an intense yellowish-green fluorescence. The zones of the faster migrating alkaloids generally exhibit a blue or yellow fluorescence under UV light. After separation by paper ionophoresis the reserpine zone is excised and eluted with 5 *N* acetic acid, and the concentration of reserpine in the eluate is determined by UV spectrophotometry.

Tablets prepared with crystalline reserpine mixed with a number of pharmaceutical excipients are assayed for reserpine by extracting the tablets with methanol, extracting a suitable blank (containing all tablet ingredients except reserpine) with methanol, deducting the absorbance, at a given wavelength, of the blank from the absorbance of the tablet extract, employing the resulting net absorbance value to calculate the concentration of reserpine in the tablet extract and hence the concentration of reserpine in the tablets. The reserpine assay of tablets prepared with a mixture of Rauwolfia alkaloids rather than with crystalline reserpine, comprises a methanol extraction of the tablets followed by paper ionophoresis of the resulting extract.

Optical rotations of reserpine samples are determined with 5 *N* acetic acid as the solvent, inasmuch as solutions of reserpine in this solvent are more stable than in chloroform. A rough assay of reserpine samples by optical rotation is used to check the assay by UV spectrophotometry.

RESERPINE ASSAY PROCEDURES

UV Absorption Characteristics of Reserpine.—As shown in Fig. 1, the UV absorption curve of a solution of pure reserpine[1] (found, C, 65.19; H, 6.67; N, 4.61; calcd. for $C_{33}H_{40}O_9N_2$, C, 65.11; H, 6.62; N, 4.60) in methanol exhibits the following characteristics: λ min. 246 mμ ($E^{1\%}_{1\,cm.}$ 159), λ max. 267–268 mμ ($E^{1\%}_{1\,cm.}$ 278), λ min. 288 mμ ($E^{1\%}_{1\,cm.}$ 169),

* Received June 28, 1954, from the Warner-Chilcott Research Laboratories, 113 West 18th Street, New York 11, N. Y.

The authors are greatly indebted to R. A. Arzapallo for valuable technical assistance and for help in the elaboration of the condensed assay formulas.

[1] Sample furnished by Dr. John Shavel, Jr., of these Laboratories.

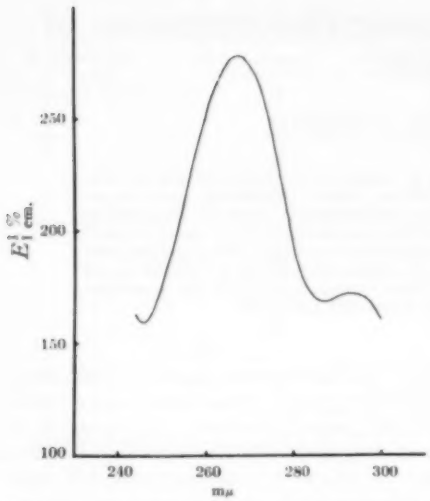

Fig. 1.—UV absorption spectrum of pure reserpine in methanol

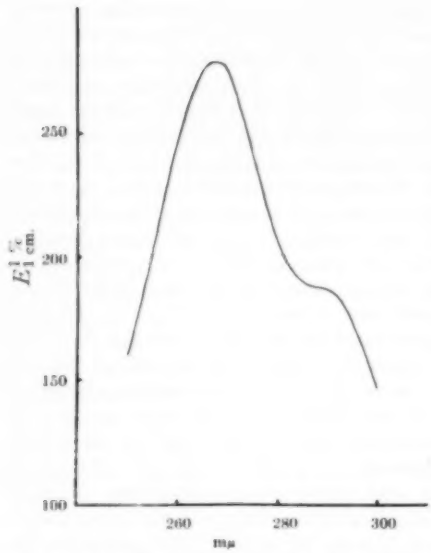

Fig. 2.—UV absorption spectrum of pure reserpine in 5 *N* acetic acid

λ max. 293–295 mμ ($E^{1\%}_{1\,cm.}$ 172), absorbance at 275 mμ equals absorbance at 259.25 mμ, ratio *K* of absorbances at 267 mμ and 275 mμ equals 1.141. This curve undergoes slight changes upon exposure of the solution to air, light, or UV irradiation, whereas under these conditions the UV absorption curve of a 5 *N* acetic acid solution of reserpine (Fig. 2) undergoes no change and hence is more suitable for use in analytical work. The characteristics of such a curve are as follows: λ max. 267 mμ ($E^{1\%}_{1\,cm.}$ 278), shoulder at 290 mμ ($E^{1\%}_{1\,cm.}$ 187), $E^{1\%}_{1\,cm.}$ 147 at 300 mμ, absorbance at 275 mμ equals absorbance at 259.25 mμ, ratio *K* of absorbances at 267 mμ and 275 mμ equals 1.147. In 5 *N* acetic acid Beer's law is obeyed for reserpine concentrations ranging from 5 to 30 mg. per liter, which range corresponds to the absorbance range 0.14 to 0.84 at 267 mμ.

Assay of Reserpine Samples Containing No UV Absorbing Contaminant.—Samples of reserpine containing no UV absorbing contaminant may be assayed in 5 *N* acetic acid solution by means of the following formula:

$$\%\ \text{Reserpine in sample} = \frac{E^{1\%}_{1\,cm.}\ (\text{of sample at 267 m}\mu) \times 100}{E^{1\%}_{1\,cm.}\ (\text{of pure reserpine at 267 m}\mu)} \quad (1)$$

Assay of Reserpine Samples Containing a Linear, UV Absorbing Contaminant.—In the case of a reserpine sample which contains a UV absorbing contaminant whose UV absorption curve is linear (i. e., plot of absorbance *vs.* wavelength is a straight line) over the range 259.25 to 275 mμ, the observed absorbance (D_λ) at any wavelength λ mμ of a 5 *N* acetic acid solution of the sample may be corrected for the slope *m* and height of general absorption *h* imparted by the linear "irrelevant absorption," in a manner similar to that discussed by Morton and Stubbs (10–12) in connection with studies in vitamin A. Accordingly, the corrected absorbance D_λ (corr.) at λ mμ due to reserpine may be derived from the following equation:

$$D_\lambda(\text{corr.}) = D_\lambda - m - h \quad (2)$$

where *m* and *h* are defined as follows:

$$m = \frac{(275 - \lambda)}{(275 - 259.25)}\,(D_{259.25} - D_{275}) \quad (3)$$

$$h = \frac{1.147\,D_{275} - (D_\lambda - m)}{0.147} \quad (4)$$

Substituting 267 for λ, and the values *m* and *h* indicated in Eqs. 3 and 4, Eq. 2 may be condensed to the following equation:

$$D_{267}\,(\text{corr.}) = 7.802\,D_{267} - 3.840\,D_{275} - 3.963\,D_{259.25} \quad (5)$$

The $E^{1\%}_{1\,cm.}$ may then be obtained from D_{267}(corr.) and entered in Eq. 1 to furnish the reserpine assay of the sample. The assumption of linearity for the UV absorbing contaminant may be checked by similarly correcting the observed absorbance at all points of the UV absorption spectrum of the sample between 259.25 and 275 mμ, and plotting the curve log *D* (corr.) *vs.* wavelength. Should this curve be not superimposable with the corresponding curve of pure reserpine over the same wavelength range, it may be presumed that the irrelevant absorption is nonlinear and the correction applied therefore is invalid.

Assay of Reserpine Samples Containing Known UV Absorbing Contaminants.—When a reserpine sample is contaminated by UV absorbing materials which possess nonlinear UV absorption curves, an assay for reserpine by simultaneous equations (13) may be developed provided the UV absorption

curves of all the contaminants in pure form are known. However, the practicality and accuracy of this method dimish progressively as the number of such contaminants increases beyond one. In some cases it may be possible that at a certain wavelength in the UV spectrum, the extinction coefficient of reserpine has a reasonably high value whereas that of the UV absorbing contaminant is small or negligible by comparison. For example, in 5 *N* acetic acid solution, the $E^{1\%}_{1\ cm.}$ of ajmalicine[2] (14) at 300 mμ is only about 6.5% of the $E^{1\%}_{1\ cm.}$ of pure reserpine at the same wavelength. Thus, mixtures of reserpine and ajmalicine containing up to 13% of the latter may be assayed for reserpine by absorbance measurements at 300 mμ of solutions of the mixtures in 5 *N* acetic acid, while assuming the contribution of ajmalicine to be not more than 1% of the observed absorbance values.

Assay of Reserpine Samples Containing Unknown UV Absorbing Contaminants.—In the cases where it is known or suspected that the reserpine sample to be assayed contains unknown UV absorbing contaminants or in the cases where no reliable method can be found to calculate and deduct the absorbance of known contaminants from the gross absorbance of the reserpine sample, the method of reserpine assay used comprises a preliminary separation of reserpine from the mixture by the technique of paper ionophoresis described below.

Apparatus.—The apparatus is essentially as described by Flynn and De Mayo (15), and consists of a lucite cabinet measuring 45 x 20 x 40 cm. (Fig. 3). The two inner as well as the two outer compartments are connected by rubber tubes in order to balance the liquid levels on either side of the glass support (ridge pole) thus preventing siphoning of liquid between compartments by way of the filter paper strips. The connecting tubes are closed while ionophoresis is in progress. The source of direct current is a full-wave rectifier capable of a smoothed output of 600 v. at 300 ma, which on a small load delivers about 1,000 v. The output is controlled by means of a Powerstat transformer at the input side.

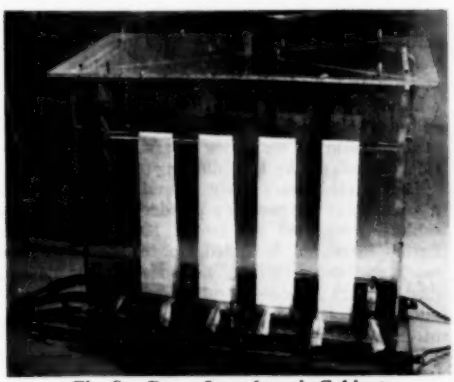

Fig. 3.—Paper Ionophoresis Cabinet

Procedure.—The filter paper used throughout this work is Whatman No. 3 MM paper obtained in rolls 4 inches wide and 600 feet long (Fisher Scientific Co.). Strips about 60 cm. long are cut from the roll, washed forty-eight hours with 5 *N* acetic acid by downward capillary flow in the "Chromatocab" chromatography cabinet (Research Equipment Corp., Oakland, Calif.) whereafter the paper is handled only with surgical rubber gloves. The washed strips are air dried and each is bisected lengthwise and trimmed at the narrow edges to give a pair of twin strips measuring 5.1 x 56 cm., one being designated *sample strip* and the other *control strip*. A solution of the submitted sample in a suitable solvent (e. g., 5 *N* acetic acid, methanol, 50% 5 *N* acetic acid-50% methanol, dioxane) is accurately delivered from a Rheberg microburet (New York Laboratory Supply Co., Inc., New York, N. Y.) onto a lead pencil line (origin) drawn equidistant from the two short edges on the sample strip. The solution is applied to the paper as a band not more than 0.4 cm. wide and 3 cm. long, in increments of about one microliter, the solvent being evaporated by means of a gentle stream of compressed air following each application. The control strip is set aside and protected from contamination. As a duplicate in the determination, a second pair of twin filter paper strips is prepared and the sample strip is spotted with the solution of the sample and the control strip is set aside as before. If an estimate of the reserpine content of the sample is available, the amount of sample applied to each sample strip should, if possible, correspond to not less than about 40 μg. of reserpine (e. g., if estimate is 2% reserpine on sample, about 40 μl. of a 5% solution of the sample is applied to each sample strip). Each of the four strips is clearly marked in order to relate it to its appropriate twin, e. g., Sample No. 1, Control No. 1, Sample No. 2, Control No. 2. Each of the four compartments of the ionophoresis cabinet is filled with 700 ml. of 5 *N* acetic acid. Then each of the duplicate sample strips is creased lightly along the origin and draped over the ridge pole (set at 26 cm. above the acetic acid level) in the ionophoresis cabinet in such a way that the strip is in contact with the ridge pole at the origin, on the reverse side of the paper, and that the ends of the strip dip into the acetic acid in the inner cells on either side of the ridge pole. The strips are moistened with 5 *N* acetic acid applied from a pipet, working upward on both sides of the apex until a paper area of only about 1 cm. wide on either side of the origin remains dry. The solvent is then allowed to ascend to the apex of the strip by capillarity. The lid of the cabinet is replaced and there is applied a potential of 300 v. for sixteen hours (for crude fractions) or 600 v. for four hours (for the more refined fractions) whereafter the strips are removed from the cabinet and air dried for about forty-five minutes.

Upon visualization of the resolved pattern of alkaloids on each sample strip under a 366 mμ UV lamp (Research Equipment Corporation, Oakland, Calif.), the entire reserpine zone, which has consistently been the slowest migrating Rauwolfia alkaloid having a yellowish-green fluorescence under UV light, is carefully circumscribed by means of a lead-pencil line. At this point the control strip corresponding to each sample strip is carefully

[2] Sample furnished by Dr. John Shavel, Jr., of these Laboratories.

superimposed with the latter and an accurate tracing is made upon the control strip of the pencil line circumscribing the reserpine zone on the sample strip. A solution of pure reserpine is spotted onto the control strip within the area defined by the tracing, the solution being accurately delivered from a Rheberg microburet, and the volume spotted corresponding to about 40 μg. of pure reserpine (e. g., 40 μl. of a 0.1% solution in 5 *N* acetic acid). Thereafter the reserpine zones in each of the sample and control strips are cut out along the pencil lines defining the zones and separately cut up into sections about 0.5 cm.² (handle with rubber gloves!) and placed into suitably labeled glass-stoppered, 15 x 125-mm. test tubes.

The four test tubes representing a determination in duplicate are then placed (with stoppers removed) into a vacuum oven at 60° at about 30 mm. pressure for thirty minutes, whereafter 4.0 ml. of 5 *N* acetic acid is pipetted into each tube and the stoppered tubes are shaken gently and allowed to stand overnight at about 5°. The tubes are then allowed to warm up to room temperature, are shaken gently, centrifuged, and the absorbances of each clear supernate are measured in a Beckman spectrophotometer, model DU, at the following wavelengths: 259.25, 267, and 275 mμ. The solvent used in the reference cell is a sample of 5 *N* acetic acid especially set aside for this purpose from the same lot that was used for elution of the reserpine from the filter paper strips.

Calculations.—In all cases pertaining to the assay by paper ionophoresis of extracts or fractions dederived from *R. serpentina* or *R. micrantha*, a correction for linear irrelevant absorption is applied to absorbance readings of all eluates of sample strips and control strips, in accordance with Eqs. 2, 3, and 4 to obtain D_{267}(corr.); the reserpine concentration in each eluate is then derived by dividing D_{267}(corr.) by the $E^{1\%}_{1\text{ cm.}}$ of pure reserpine in 5 *N* acetic acid. In arriving at the assay formula, a further correction is applied to the found concentration of each sample strip eluate, by adding thereto a value *a* which represents the difference (in μg. per ml.) between the found and theoretical concentrations of reserpine in the corresponding control strip. The assay formula may now be written as follows:

For solid samples:

$$\%\text{ reserpine} = \frac{400}{W}\left[\frac{D_{267}(\text{corr.})\,(\text{sample}) \times 10^4}{E} + a\right] \quad (6)$$

For liquid samples:

$$\text{Gm. reserpine per 100 ml.} = \frac{0.4}{V}\left[\frac{D_{267}(\text{corr.})\,(\text{sample}) \times 10^4}{E} + a\right] \quad (7)$$

where

E = $E^{1\%}_{1\text{ cm.}}$ of pure reserpine in 5 *N* acetic acid
W = weight in μg. (solid basis) of sample applied to sample strip
V = volume in μl. applied to sample strip
a = concentration correction in μg. per ml. defined as follows:

$$a = \left[\frac{X}{4} - \frac{D_{267}(\text{corr.})\,(\text{control}) \times 10^4}{E}\right] \quad (8)$$

where X = weight in μg. (solid basis) of pure reserpine applied to control strip.

Combining Eqs. 2, 3, 4, 6, 8 and Eqs. 2, 3, 4, 7, 8, there are obtained condensed Eqs. 9 and 10, respectively.

$$\%\text{ reserpine} = \frac{1 \times 10^7}{WE}\,[XE \times 10^{-6} - 1.585\,(D_A - D'_A) + 3.121\,(D_B - D'_B) - 1.536\,(D_C - D'_C)] \quad (9)$$

$$\text{Gm. reserpine per 100 ml.} = \frac{1 \times 10^4}{VE}\,[XE \times 10^{-6} - 1.585\,(D_A - D'_A) + 3.121\,(D_B - D'_B) - 1.536\,(D_C - D'_C)] \quad (10)$$

where:

D_A = observed absorbance of sample eluate at 259.25 mμ
D_B = observed absorbance of sample eluate at 267 mμ
D_C = observed absorbance of sample eluate at 275 mμ
D'_A = observed absorbance of control eluate at 259.25 mμ
D'_B = observed absorbance of control eluate at 267 mμ
D'_C = observed absorbance of control eluate at 275 mμ

Assay of Reserpine Samples by Optical Rotation.—The optical rotation of a 1% solution of a reserpine sample in 5 *N* acetic acid (filtered through a sintered glass filter, if turbid) is determined in a 2-dm. polarimeter tube. The specific rotation of pure reserpine under these conditions is −111° ± 1. If it is known that the optical activity of the contaminants is negligible, the assay of the sample may be obtained from the ratio of its specific rotation (in 5 *N* acetic acid) to −111°. For example, ajmalicine has a specific rotation of −5.9° in 5 *N* acetic acid. Thus for reserpine samples containing ajmalicine as a contaminant up to about 17%, the contribution of ajmalicine to the specific rotation of the mixture is not greater than 1% and is therefore negligible.

Assay of Reserpine Tablets.—Reserpine tablets containing 0.1 to 0.25 mg. of reserpine per tablet admixed with such excipients as starch, methylcellulose, lactose, stearic acid, and magnesium stearate, and in some cases hexamethonium chloride, are assayed by the following procedure.

Eleven tablets are finely ground in a mortar and a quantity of the resulting powder equivalent to 10 tablets is weighed out into a 100-ml. volumetric flask. Methanol is added up to the calibration mark and the remaining air space in the flask is flushed with nitrogen, and the flask is stoppered and shaken vigorously for not less than ten minutes. About 10 ml. of the resulting suspension is centrifuged in a glass-stoppered tube and the optical density at 267 mμ (D_T) of the clear supernatant is determined within thirty minutes from the time methanol was added to the ground tablet powder. A methanol extract of the appropriate blank (same composition as tablets except that reserpine

is omitted) equivalent to 10 tablets, is prepared exactly as indicated for the tablets, and its absorbance at 267 mμ (D_{BL}) is determined. The weight of the reserpine in the tablets is then obtained from the following formula:

$$\text{Mg. reserpine per tablet} = \frac{(D_T - D_{BL})F \times 100}{E} \quad (11)$$

where E is the $E^{1\%}_{1\text{ cm.}}$ at 267 mμ of pure reserpine in methanol and F is a purity correction factor which represents the ratio of the known assay (as determined by one of the UV methods described in the foregoing sections) of the reserpine lot used in manufacture of the tablets, to the observed assay derived directly from the uncorrected absorbance at 267 mμ of the same reserpine lot in methanol solution. In cases where pure reserpine is used in tablet manufacture, $F = 1$.

In the event that suitable blanks are not available, the reserpine tablets may be assayed by the method indicated below for tablets containing mixed Rauwolfia alkaloids, or by applying a correction for linear UV absorbing contaminants (all tablet excipients mentioned above behave as such) in accordance with Eqs. 2–4, except that all absorbances relate to the methanol extract of 10 tablets, and in Eq. 4, the numbers 1.147 and 0.147 are replaced by 1.141 and 0.141, respectively, to conform with the value of the ratio K (ratio of D_{257} to D_{275}) for methanol solutions of pure reserpine. The value derived for D_{257} (corr.) for the methanol extract of 10 tablets is then used to determine the reserpine assay of tablets in accordance with the following condensed equation:

$$\text{Mg. reserpine per tablet} = 100/E\ [8.093\ D_{267} - 3.983\ D_{275} - 4.111\ D_{259.25}] \quad (12)$$

The reserpine assay method employing the tablet blank is preferred to the method employing Eq. 12, inasmuch as slight amounts of decomposition resulting from failure to protect adequately the methanol extracts with nitrogen, may lead to appreciable error in the use of Eq. 12.

Assay of Reserpine in Tablets Containing Mixed Rauwolfia Alkaloids.—Eleven tablets are finely ground and a quantity of the resulting powder equivalent to 10 tablets is introduced into a 40-ml. capacity centrifuge tube. Ten milliliters of methanol is added to the powder and the suspension is thoroughly stirred with a glass tube through which a steady stream of nitrogen is passed. The suspension is centrifuged, and the supernatant is decanted and kept under nitrogen. A further 10 ml. of methanol is added to the residual solid in the centrifuge tube and the extraction procedure is repeated. After a total of seven such extractions, the combined extracts are filtered through a sintered glass filter, the filter is washed with a few milliliters of methanol and the combined filtrates are evaporated to dryness under vacuum at room temperature. The residual solid is dissolved in methanol and made up to 5 ml. with methanol in a volumetric flask. The resulting solution is centrifuged in a glass-stoppered tube to remove any slight turbidity that may be present and the reserpine concentration in the clear supernatant is determined by the paper ionophoresis method (the methanol extract should be kept under nitrogen at 5° and assayed on the same day if possible). In order to prevent charring of the paper during ionophoresis, the volume of extract applied to the paper is not higher than about 250 μl. and the ionophoresis is run at 300 v. for eighteeen hours then at 600 v. for seven to twenty-four hours or until a satisfactory resolution of the reserpine zone is obtained. Because of the limitations on the volume of tablet extract which can be applied to the paper, this assay is impractical for tablets containing less than about 0.02 mg. of reserpine per tablet.

PRECISION OF RESULTS

In all assays described above considerable care is exercised to exclude contamination by UV absorbtion of extraneous materials such as fingermarks and sweat. Furthermore, all solutions employed in assay work are excluded from contact with rubber, polyethylene, and cork stoppers, all of which were found to introduce appreciable errors. In general the precision of the direct UV assay of crystalline reserpine containing 90% minimum reserpine is ±1.5%, and the precision of the paper ionophoresis assay for the same material is ±1.8%. Rauwolfia root extracts and alkaloidal fractions containing up to about 50% reserpine have been assayed for reserpine by paper ionophoresis with a reproducibility of about ±2.5% provided that the equivalent of not less than about 25 μg. of pure reserpine was applied to the sample strips. Thus the precision of paper ionophoresis assays of solutions containing

TABLE I.—RESULTS FROM TYPICAL SETS OF RESERPINE ASSAYS

Description of Sample	Method	Reserpine Assay Values	Mean	Standard Deviation, %
Crystalline reserpine	UV absorption corrected for linear contamination (20 mg. per liter)	94.6, 95.0, 96.8, 97.2, 91.9, 95.3, 92.7, 96.1	94.8	1.9
Pure reserpine	UV absorption corrected for linear contamination (20 mg. per liter)	98.8, 97.2, 99.3, 96.8	98.0	1.2
Pure reserpine	Paper ionophoresis	96.8, 101.3, 99.8, 97.0	98.8	2.2
Ethanol extract of *R. micrantha*	Paper ionophoresis	0.519, 0.515, 0.502, 0.500, 0.494, 0.500, 0.516, 0.501, 0.536 (Gm. per 100 cc.)	0.508	2.6
Crystalline reserpine tablets	Methanol extraction and UV absorption	0.151, 0.152, 0.151, 0.149 (mg. per tablet)	0.151	0.9

less than 0.01 Gm. reserpine per 100 ml. is lower, inasmuch as the application to the sample strips of volumes higher than 250 µl. becomes impractical. The accuracy of the ionophoresis method based on test runs with pure reserpine is ±2%. The precision of assays of tablets prepared with crystalline reserpine is about ±1%.

DISCUSSION

The quantitative determination of reserpine in samples of crystalline material which usually contain a minimum of 90% of reserpine is generally carried out by the direct UV absorption method involving a correction for background, linear, irrelevant absorption in accordance with Eq. 5. The resulting assay value is routinely compared with the assay value furnished by optical rotation measurement in 5 *N* acetic acid, and the two values are generally found to be within about 1% of their mean. In the event that such agreement is not obtained a further check is made by the paper ionophoresis method whose results are accurate to ±2% as determined by test runs with pure reserpine.

The above described procedure for the assay of reserpine tablets is based on the assumption that the UV absorption due to reserpine is derived by deducting the absorption of the excipients from the absorption of the total tablet extract, and therefore that any possible compound formation between reserpine and the excipients would have no effect upon the spectral characteristics of reserpine. The correctness of this assumption was demonstrated in all cases of tablets so assayed, by showing that the absorbance, at a given wavelength, of a synthetic mixture of the tablet blank and pure reserpine is equal to the sum of the absorbances of the blank and the reserpine measured separately.

In the paper ionophoresis assay of Rauwolfia extracts and fractions derived therefrom, the use of a correction for linear irrelevant absorption, in accordance with Eqs. 2–4, is based on the following observations:

(*a*) In the course of a number of paper ionophoresis assays of crude extracts of *R. serpentina* and *R. micrantha*, the absorbances of the sample strip eluate, at all wavelengths λ between 259.25 and 275 mµ, were corrected in accordance with Eqs. 2–4, as was done for the optical density at 267 mµ (*m* varies with λ, but *h* is the same for 267 mµ as for other values of λ). The plotted curve log *D* (corr.) *vs.* λ was invariably found to be superimposable with the corresponding curve of pure reserpine in 5 *N* acetic acid, over the range 259.25 to 275 mµ.

(*b*) Following a satisfactory resolution by paper ionophoresis of crude extracts of Rauwolfia root, the reserpine zone visualized by fluorescence under UV appears discrete and well defined (Fig. 4). Non-fluorescent zones contiguous to the reserpine zone on the anode side and on the cathode side, were excised, eluted with 5 *N* acetic acid and the resulting eluates were found to exhibit substantially linear UV absorption spectra (Fig. 5). Further, the reserpine zone itself was transferred by descending capillary elution with 5 *N* acetic acid, onto a small area of a strip of Whatman No. 1 filter paper, whereafter the transferred spot was subjected to ascending paper chromatography with 0.02 *M* citric acid/trisodium citrate buffer *p*H 3.5; various portions of the resolved paper chromatogram, other than the reserpine spot, gave 5 *N* acetic acid eluates exhibiting linear UV absorption spectra (Fig. 5). Finally 5 *N* acetic acid eluates of strips of Whatman 3 MM and Whatman No. 1 papers also gave substantially linear UV absorption spectra (Fig. 5).

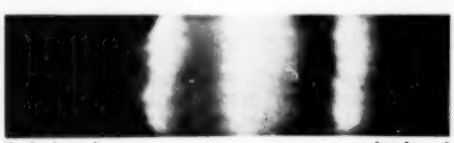

Fig. 4.—Separation of Rauwolfia Alkaloids by paper ionophoresis.

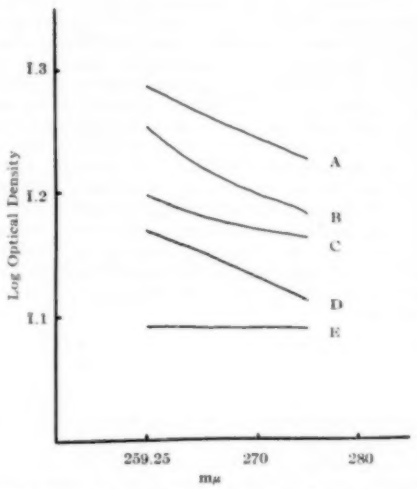

Fig. 5.—UV absorption spectra of contaminants in 5 *N* acetic acid eluates. A. Fluorescent spot, other than reserpine, on paper chromatogram. B. Nonfluorescent zone on cathode side of reserpine zone on paper ionogram. C. Nonfluorescent zone on anode side of reserpine zone on paper ionogram. D. Whatman No. 1 filter paper. E. Whatman No. 3 MM filter paper.

As previously indicated, assays by paper ionophoresis have been performed only on *R. serpentina* and *R. micrantha* and fractions derived therefrom. The validity of the described correction procedures is established periodically for new lots of the same species of root as well as for new species of root being evaluated. Although the reserpine content of Rauwolfia species is apt to vary from lot to lot, an indication is given on Table II of the order of the reserpine content of *R. serpentina* and *R. micrantha*, as determined by paper ionophoresis assay of 95% ethanolic extracts of the powdered root.[3]

[3] Extractions of root were made by Dr. John Shavel, Jr., Arnold D. Lewis, and Jerome D. Genzer.

TABLE II.—PAPER IONOPHORESIS ASSAY OF ETHANOLIC EXTRACTS OF RAUWOLFIA ROOTS

Species of Root	Order of Reserpine Concentration— On Sample (Dry Residue), %	On Root, %
R. serpentina	1.7	0.17
R. micrantha	1.4	0.06

Experiments on the assay of a given reserpine-containing sample, by paper ionophoresis (four-hour and sixteen-hour runs) revealed that substantially the same reserpine assay value was obtained for the sample regardless of whether or not the control strip containing pure reserpine was subjected to ionophoresis along with the sample strip. Subsequent to this observation the introduction of control strips into the ionophoresis cabinet was therefore routinely omitted.

Experiments on paper ionophoresis assays using pure reserpine (40 μg.) on the sample strip as well as on the control strip revealed that substantially the same assay value was obtained: (*a*) whether the excised area of the reserpine zone on sample and control strips was 2 x 5 cm. or 4 x 5 cm., and (*b*) whether the quantity of pure reserpine applied to the control strip was 20 μg., 40 μg., 80 μg., or 120 μg.

Thus within the limits indicated, the area of paper excised with the reserpine zone is not critical, and the value *a* which represents the amount of reserpine not eluted from the paper, is independent of the quantity of pure reserpine applied to the control strip.

The assay of reserpine by paper ionophoresis may be susceptible to certain simplifying changes especially in regard to alternative, more direct techniques, such as densitometry or fluorophotometry (16), for measuring the quantity of reserpine on the resolved paper ionogram. In any event it appears probable that assays of reserpine in the presence of other Rauwolfia alkaloids will generally continue to comprise a preliminary separation of reserpine from the other alkaloids present. A number of polarographic experiments were run (using the Fisher Elecdropode with mercury anode) in an effort to obviate such a separation; however, although satisfactory polarograms were obtained with pure reserpine ($E_{1/2}$ *vs. S. C. E.* at −2.18 v. and −2.30 v. in 75% aqueous dioxane containing 0.175 *M* tetrabutylammonium iodide), the polarograms given by Rauwolfia extracts and impure fractions of reserpine, with and without added pure reserpine, showed relatively high residual and limiting currents and poorly defined waves leading to unreliable diffusion current measurements.

In its present form, the paper ionophoresis method of assay appears to be impractical for the quantitative determination of reserpine in biological fluids, inasmuch as the reserpine concentration in such fluids will be below the sensitivity limits of the method. It is believed, however, that the paper ionophoresis technique will prove helpful in the investigation of more suitable methods for the assay of reserpine in biological fluids.

Finally it is believed that the technique of paper ionophoresis in 5 *N* acetic acid of Rauwolfia alkaloids will be of value in the classification studies of Rauwolfia species presently being conducted by other investigators (17, 18).

SUMMARY

1. Methods are described for the quantitative evaluation of reserpine in crude extracts of certain species of Rauwolfia root and in fractions obtained therefrom in varying degrees of purity.

2. Methods are also described for the reserpine assay of tablets prepared either with crystalline reserpine or with mixtures of Rauwolfia alkaloids.

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The Influence of Quaternary Ammonium Compounds in the Assay of Tyrothricin Antibiotics*

By LESTER L. NISONGER

Quaternary ammonium compounds are useful as solubilizing agents for the tyrothricin antibiotics. They are also strong germicidal agents and may introduce a potentiating effect in the microbial assay of the antibiotic. The influence of cetylpyridinium chloride in the assay of methylol gramicidin has been investigated and treated statistically. The findings emphasize the importance of knowing the exact concentration of quaternary present in the assay mixture.

THE ANTIBIOTIC tyrothricin U. S. P. is a natural mixture containing at least two distinct antibacterial substances which can be separated by a relatively simple process. The properties, usefulness, limitations of tyrothricin have been reviewed by Henderson (1). One component, tyrocidine, represents at least 60 per cent of the mixture, while gramicidin ranges from 20 to 25 per cent. Both are crystalline polypeptides. They are relatively water insoluble, are precipitated by salts, are hemolytic, and are toxic except for oral or topical use.

Lewis, *et al.* (2), and others (3, 4), have prepared and investigated the methylol derivative of gramicidin and found it to be much less hemolytic and toxic, but to be very little changed in antibiotic activity from gramicidin itself. In the pharmaceutical use of methylol gramicidin, it has been found desirable to employ the quaternary ammonium compound, cetylpyridinium chloride[1] for its solubilizing effect. This cationic, surface-active agent is also a potent germicide (5, 6). Certain combinations of cetylpyridinium chloride and methylol gramicidin were found to give high assay values, due to the action of the cetylpyridinium chloride on the test organism, *streptococcus* sp., Lancefield group D, strain No. H69D_5. Attempts to eliminate this interference by selective inactivation of the cetylpyridinium chloride were not successful. It has been observed that germicidally inactivated cetylpyridinium chloride often will exhibit bacteriostatic activity (7). Attempts to separate the two compounds by extraction likewise were unsuccessful, since the solubility characteristics of the methylol gramicidin were altered when in combination with cetylpyridinium chloride. It was necessary, therefore, to determine the exact degree of influence that cetylpyridinium chloride would exert in the methylol gramicidin assay.

METHOD

In this study, five levels of methylol gramicidin (representing 40, 60, 80, 100, and 120% of the 1:10,000 concentration which is commonly used) were combined with seven levels of cetylpyridinium chloride (ranging from 1:5,000 to 1:1,364). One to four assays were run for each of the resulting thirty-five combinations. The assay method employed is a turbidimetric serial dilution test, similar to the U. S. P. method (8). The end point is that concentration which inhibits growth equal to 50% light transmission when read in a Lumetron colorimeter, Model 402-E, with an M-660 filter. A laboratory reference methylol gramicidin was used as the standard.

RESULTS

It was immediately apparent that the combinations assayed should be considered from a ratio, rather than an actual concentration point of view.

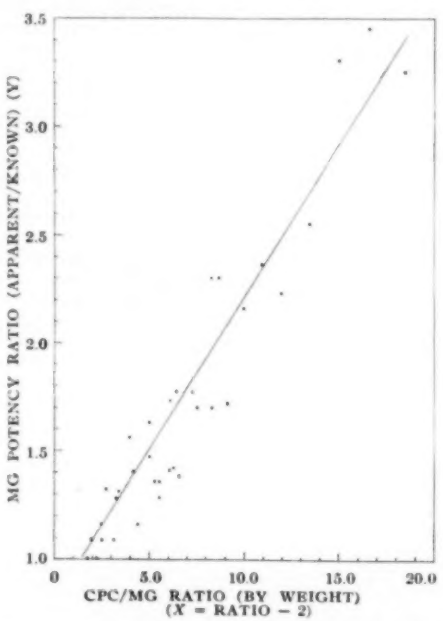

Fig. 1.—Scatter diagram showing relationship between CPC/MG ratio and MG potency.

*Received April 5, 1954, from the Department of Microbiology, Research Laboratories, The Wm. S. Merrell Company, Cincinnati, Ohio.

[1] Cetylpyridinium chloride (Ceepryn® brand) produced by The Wm. S. Merrell Company.

TABLE I.—SUMMARY OF DATA SHOWING INFLUENCE OF CETYLPYRIDINIUM CHLORIDE IN THE POTENCY ASSAY OF METHYLOL GRAMICIDIN

CPC[a]	MG[b], %	CPC/MG Weight Ratio	MG Potency, % By Assay	MG Potency, % From Curve[c]	Assay Error, %
1:1,364	120	6.10	208	198	+5.5
1:1,500	120	5.55	154	188	−17.9
1:1,665	120	5.00	196	180	+8.8
1:1,875	120	4.44	140	169	−17.1
1:3,000	120	2.77	159	140	+13.5
1:4,000	120	2.18	117	132	−11.3
1:5,000	120	1.67	121	124	−2.4
1:1,364	100	7.25	177	181	−2.2
1:1,500	100	6.66	138	173	−20.2
1:1,665	100	6.05	141	165	−17.0
1:1,875	100	5.33	136	154	−13.2
1:3,000	100	3.33	128	125	+2.4
1:4,000	100	2.50	109	114	−4.3
1:5,000	100	2.00	109	106	+2.8
1:1,364	80	9.15	138	167	−17.3
1:1,500	80	8.33	136	158	−13.9
1:1,665	80	7.50	136	148	−8.1
1:1,875	80	6.66	142	135	+5.1
1:3,000	80	4.16	125	110	+13.6
1:4,000	80	3.15	87	98	−11.2
1:5,000	80	2.50	93	91	+2.1
1:1,364	60	12.1	134	150	−10.6
1:1,500	60	11.0	142	141	+0.7
1:1,665	60	9.95	130	132	−1.5
1:1,875	60	8.85	138	121	+14.0
1:3,000	60	5.53	82	94	−12.7
1:4,000	60	4.15	85	82	+3.6
1:5,000	60	3.30	79	76	+3.9
1:1,364	40	18.3	130	136	−4.4
1:1,500	40	16.6	138	125	+10.4
1:1,665	40	15.0	132	117	+12.8
1:1,875	40	13.3	102	108	−5.5
1:3,000	40	8.33	92	79	+16.4
1:4,000	40	6.25	57	67	−14.9
1:5,000	40	5.00	59	60	−1.7
				Av.	9.2

[a] Cetylpyridinium chloride.
[b] Methylol gramicidin, values based on 100% equals 1:10,000.
[c] Derived from Fig. 1.

This is logical, since the assay end point is always a dilution of the original mixture. The ratio of concentrations, however, remains unchanged In this series the cetylpyridinium chloride to methylol gramicidin ratios, by weight, ranged from 18.3:1 to 1.67:1. As shown in Fig. 1, the assay values plotted in a scatter diagram indicate a straight-line relationship. It was expedient to make a statistical solution by the method of least squares (9), in which the line of trend is represented by the formula: $Y = a + bX$. X represents the CPC/MG ratio, grouped into classes having a ratio interval of 1.0 and a ratio of origin of 2.0 (that is, for $X = 0$, the class interval is 2.0 and this includes all ratios between 1.6 and 2.5). Y represents the MG potency ratio, apparent/known.

From the experimental data in Table I, the line of trend formula becomes: $Y = 1.086 + 0.1409\ X$. For example, in a mixture having 1:10,000 methylol gramicidin and 1:2,000 cetylpyridinium chloride, the CPC/MG ratio would be 5.0, the X value is 3.0 and Y becomes 1.509. In this case the MG assay value to be expected would be 151% of the theoretical 1:10,000. The standard error of estimate in Fig. 1 is 0.19.

The experimental findings are summarized in Table I. The data show that, in the case of a product with 1:1,500 cetylpyridinium chloride, even a 10% manufacturing excess (1:1,364) or loss (1:1,665) would affect the expected antibiotic potency.

SUMMARY

1. The potentiating effect of cetylpyridinium chloride in the assay of methylol gramicidin has been determined.

2. A combination having more than one and one-half times, by weight, of cetylpyridinium chloride as methylol gramicidin will alter the microbial assay results.

3. The exact concentration of cetylpyridinium chloride should be known to interpret accurately the microbial assay results.

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The Physics of Tablet Compression*

VI. Compressional Behavior of Phenacetin and Methacetin Granulations

By L. N. ELOWE†, T. HIGUCHI, and L. W. BUSSE

A comparative study was made of the compressional behavior of methacetin and phenacetin granulations. Phenacetin tablets made at high compressional forces from a granulation containing a low proportion of partially hydrolyzed starch were physically defective. Incorporation of a higher percentage of partially hydrolyzed starch for binding purposes as well as other different binding agents in phenacetin formulations resulted, on compression, in tablets free from capping or splitting. The compressional behavior of a methacetin granulation was found to be different from that of a phenacetin granulation containing approximately the same proportion of the same binding agent.

MANY PHARMACEUTICAL manufacturers encounter considerable difficulties in the making of phenacetin tablets containing low percentages of starch paste as binding agent. Splitting and capping frequently occur in such tablets during the process of compression. To overcome these difficulties, formulations based on high concentrations of binding agents such as acacia, methyl cellulose, cellulose acetate hydrogen phthalate, and partially hydrolyzed starch have been used in the manufacture of phenacetin tablets.

Earlier work had shown a general qualitative similarity among several formulations when subjected to different degrees of compression (1, 2). In the present study, the effect of maximal compressional force on the physical properties of tablets obtained from a commonly used phenacetin granulation and the other phenacetin formulas containing high proportions of binding agents has been investigated. Efforts were made to compare the compressional behavior of these different formulations with each other and with the systems studied earlier.

Tablets were also prepared from methacetin (*p*-methoxyacetanilide), a compound closely related in structure to phenacetin for the purpose of comparing the compressional behavior of these two compounds.

Hardness, true and apparent densities, porosity and specific surface area measurements were obtained in these studies of tablets compressed from phenacetin in combination with different binding agents. The binding agents used were as

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The paper is based in part on a dissertation submitted to the Graduate School of the University of Wisconsin by L. N. Elowe in partial fulfillment of the requirements for the degree of Doctor of Philosophy.
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follows: (*a*) 4.7 and 20 per cent partially hydrolyzed starch, (*b*) 20 per cent acacia, (*c*) 20 per cent methyl cellulose, and (*d*) 8 per cent cellulose acetate hydrogen phthalate.

The methacetin tablets were made from a granulation containing 5.2 per cent partially hydrolyzed starch.

RESULTS AND DISCUSSION

The occurrence of splitting and capping in phenacetin tablets can apparently be attributed in part to the hardness of phenacetin particles. This can be seen from specific surface area measurements.

The surface area of phenacetin tablets containing 4.7% partially hydrolyzed starch showed a similar qualitative relationship with compressional force as obtained in earlier studies of various other formulations (1, 2). Figure 1 shows a maximum surface area for these tablets at 2,500 lb. compressional force. At this point the surface area is three times that of the phenacetin granules from which the tablets are made. Higher increases in surface areas were obtained for the formulations of the earlier studies (1, 2).

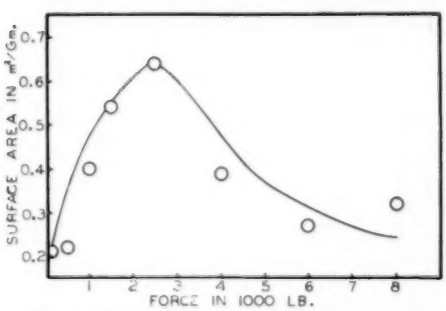

Fig. 1.—The effect of compressional force on the specific surface area of phenacetin tablets containing 4.7% partially hydrolyzed starch.

It can be seen from Table I that phenacetin tablets with the lower percentage of partially hydrolyzed starch are more resistant to an increase in their apparent density, as a result of rising compressional force, than the tablets made from the other four phenacetin formulations.

This means that the phenacetin tablets containing the higher percentage of partially hydrolyzed starch and the new binding agents are more easily compressed, a fact reflected in their value of porosity as shown in Fig. 2. At 500 lb. compressional force, the porosity of the tablets under comparison shows increasing values in the following order: phenacetin with 4.7% partially hydrolyzed starch, phenacetin with 20% partially hydrolyzed starch, phenacetin with methyl cellulose, phenacetin with cellulose acetate hydrogen phthalate, phenacetin with acacia. In all cases, porosity diminishes with increasing compressional force, declining sharply in the beginning and leveling off at higher forces. The range of this decrease, however, is a characteristic of the individual formulation and is therefore not necessarily of the same magnitude in all cases. This is clearly seen at 8,000-lb. force level where the tablets

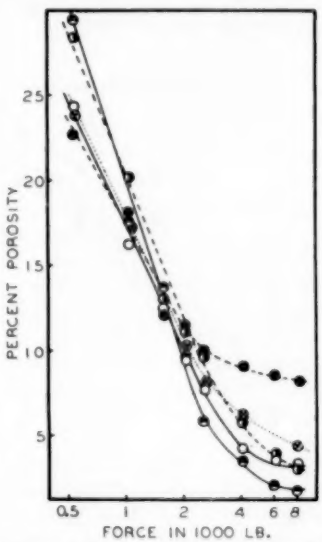

Fig. 2.—The effect of compressional force on the porosity of various tablets. ● phenacetin with 4.7% partially hydrolyzed starch, ⊗ phenacetin with 20% partially hydrolyzed starch, ◓ phenacetin with acacia, ○ phenacetin with methyl cellulose, ◑ phenacetin with cellulose acetate hydrogen phthalate.

TABLE I.—APPARENT DENSITIES[a] OF PHENACETIN TABLETS

Force	Binding Agents: Partially Hydrolyzed Starch 4.7%	Partially Hydrolyzed Starch 20%	Acacia	Methyl Cellulose	Cellulose Acetate H-phthalate
500	1.012	1.011	0.922	0.971	0.925
1,000	1.074	1.102	1.077	1.077	1.034
1,500	1.124	1.157	1.127	1.150	1.128
2,000	1.156	1.195	1.180	1.160	1.151
2,500	1.174	1.225	1.229	1.181	1.171
4,000	1.185	1.254	1.260	1.229	1.220
6,000	1.188	1.265	1.278	1.235	1.244
8,000	1.191	1.271	1.283	1.239	1.255

[a] Apparent densities are given in Gm./cc.

show values of porosity in an order which is the reverse of that exhibited at the initial compressional force of 5,000 lb.

The rate of decrease of tablet porosity with increasing compressional force is apparently a measure of the effectiveness of the binding agent used. This, in turn, is a criterion of the ease with which tablets can be compressed and reflects on the physical properties of the tablets after compression. In the case of phenacetin with 4.7% partially hydrolyzed starch, this rate is rather slow, the tablets still retaining the comparatively high porosity of 8.5% at the 8,000 lb. force level. On the other hand, it is faster in the case of the other phenacetin tablets, the phenacetin with acacia showing the most rapid decrease and, consequently, the best compressibility. This is borne out in the values of hardness of these tablets. At corresponding compressional levels, it is seen from Fig. 3 that the phenacetin with acacia tablets are the hardest and the phenacetin tablets with the lower percentage of partially hydrolyzed starch the softest of the five.

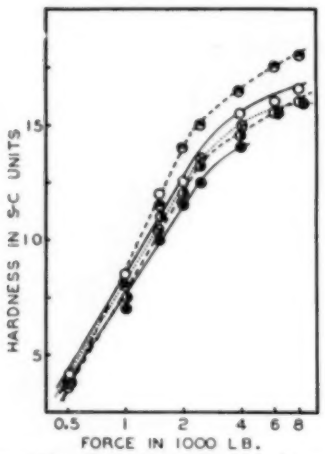

Fig. 3.—The effect of compressional force on the hardness of various tablets. ● phenacetin with 4.7% partially hydrolyzed starch, ⊗ phenacetin with 20% partially hydrolyzed starch, ◓ phenacetin with acacia, ○ phenacetin with methyl cellulose, ◑ phenacetin with cellulose acetate hydrogen phthalate.

Methacetin tablets were expected to show similar characteristics to those of phenacetin in view of the similarity in structure of the compounds. However, in this study, no such correlation between structure and compressional behavior became evident. Absence of capping or splitting in the methacetin tablets was noticeable. This is significant due to the fact that the methacetin granulation contained approximately the same proportion of the binding agent used in the case of the phenacetin granulation that yielded defective tablets. The better compressibility of the methacetin tablets is again reflected in the rate of decrease of their porosity with ascending levels of compressional force. Figure 4 shows that the decrease is faster in the case

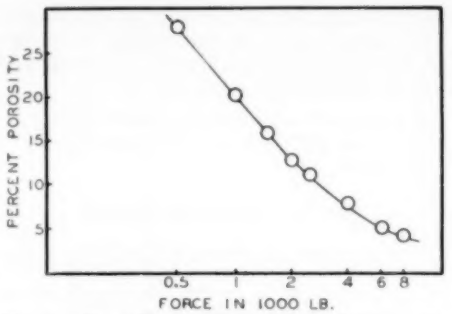

Fig. 4.—The effect of compressional force on the porosity of methacetin tablets.

of methacetin than it is in the case of phenacetin with the lower percentage of the same binding agent. Also, a smaller porosity is retained by the methacetin tablets at 6,000–8,000 lb. force levels. The relation of hardness and porosity is once again demonstrated here. At corresponding force levels, the hardness of methacetin tablets can be seen from Fig. 5 to be greater when compared to that of the phenacetin tablets with the 4.7% partially hydrolyzed starch.

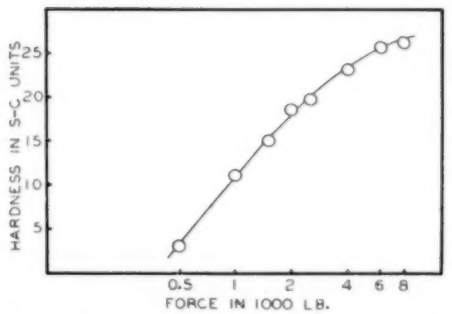

Fig. 5.—The effect of compressional force on the hardness of methacetin tablets.

The only granulation resulting in defective tablets at high degrees of compression was phenacetin with the lower percentage of partially hydrolyzed starch as binding agent. Tablets made from all other formulations were conspicuously free from such defects. This behavior can perhaps be attributed to two factors: (*a*) the high resistance to crushing offered by the phenacetin particles and (*b*) the insufficiency of the binding agent used.

It is apparent that as the compressional force increases the particles of which a tablet is composed are brought nearer to each other. This of course results in a smaller percentage of void space. At an optimum degree of compression that is characteristic for each individual granulation; the particles cannot be forced any closer to each other. At this stage, any increase in compressional force will cause one of two things: (*a*) the fragmentation of the particles and consequently still lower porosity values

or (*b*) the deformation of the particles under pressure if they are resistant to crushing.

The deformation under high pressures of particles that resist crushing can be either permanent or reversible as demonstrated by various workers in the analogous field of powder metallurgy (3–6). Which of the two will occur depends upon the nature of the particles and the kind and percentage of the binding agent present.

Phenacetin particles appear to be very resistant to crushing, undergoing an elastic deformation at high compressional forces. In the case of the granulation containing 4.7% partially hydrolyzed starch, the binding agent is apparently not sufficient to overcome the tendency by the deformed particles to assume their initial shapes as the force is released. Thus, a sudden removal of the pressure causing this kind of elastic reversible deformation results in a springback of the particles. This finds relief in a form of capping or splitting. A 20% partially hydrolyzed starch in the phenacetin granulation seems to be of sufficient binding ability to counterbalance the tendency of the deformed particles to regain their original orientation upon release of the pressure. Therefore, this granulation yields tablets free from physical defects. The same explanation can be applied to the phenacetin with acacia, with methyl cellulose, and with cellulose acetate hydrogen phthalate—the elasticity of the phenacetin particles being overcome by the different binding agents in the proportions in which they are present.

EXPERIMENTAL

Preparation of Formulations.—Two different formulations of phenacetin were prepared with starch paste. A 10% starch paste was used in one and a 20% in the other. When dried at 110° F. for four hours, the granules contained 4.7% partially hydrolyzed starch in the first and 20% in the second formulation.

Phenacetin with acacia, with methyl cellulose, and with cellulose acetate hydrogen phthalate formulations were obtained from the Smith, Kline and French Laboratories. They were prepared by spray-drying suspensions of phenacetin in solutions of the different binding agents. The assay showed that the phenacetin with acacia and with methyl cellulose each contained 80% phenacetin while the formulation with cellulose acetate hydrogen phthalate had 92% phenacetin.

Methacetin granulation was prepared with 10% starch paste. After drying at 110° F. for four hours, it contained 5.2% partially hydrolyzed starch.

Compression into Tablets.—A mechanical lever machine, in conjunction with a set of $^3/_8$-inch die and punches, was used for the compression of all tablets at various force levels (7).

Measurement of Physical Properties of Tablets.—(*a*) True densities were measured by means of a helium densitometer (7), (*b*) apparent densities by means of a specially designed tablet pycnometer (1, 7), (*c*) hardness by the Strong-Cobb tablet hardness tester (1), and (*d*) surface area on the B. E. T. low-temperature nitrogen adsorption apparatus (1).

Weight of Formulations Compressed into Tablets.—The true densities obtained by helium densitometry were utilized for the calculation of the weights of the different formulations that would yield a constant true volume in all tablets. Table II shows the true densities and the weights compressed into tablets in these studies.

TABLE II.—TRUE DENSITIES AND WEIGHTS COMPRESSED INTO TABLETS OF DIFFERENT FORMULATIONS

Formulation	True Density, Gm./cc.	Wt. Compressed, Gm.
Phenacetin with 4.7% partially hydrolyzed starch	1.297	0.3300
Phenacetin with 20% partially hydrolyzed starch	1.331	0.3380
Phenacetin with acacia	1.306	0.3320
Phenacetin with methyl cellulose	1.284	0.3265
Phenacetin with cellulose acetate hydrogen phthalate	1.295	0.3290
Methacetin with 5.2% partially hydrolyzed starch	1.281	0.3255

SUMMARY

1. The compressional behavior of different formulations made from phenacetin with various binding agents was studied.

2. A comparative study of methacetin granulation was also carried out.

3. All formulations studied gave the same qualitative relationships between maximal compressional force and the different physical properties of the tablets made from them.

4. Apparent density and hardness of tablets were found to vary directly, and porosity inversely, with the logarithm of the maximal compressional force.

5. Phenacetin tablets made from a granulation containing 4.7% partially hydrolyzed starch as binding agent were physically defective when compressed at high force levels. The capping was absent when phenacetin formulations containing a higher percentage of the same binding agent, or other binding agents, were compressed.

6. No correlation between structure and compressional behavior was obtained in the comparative study of phenacetin and methacetin granulations containing approximately the same proportion of the same binding agent.

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The Safety and Fate of Potassium Sodium Copper Chlorophyllin and Other Copper Compounds*

JOS. W. E. HARRISSON, SIMON E. LEVIN, and BERNARD TRABIN

The growth rate, survival, blood and urine factors, and ability to conceive, were normal in albino rats, who were fed up to three percent of potassium sodium copper chlorophyllin in their diets over their life span. There was no other indication of toxicity. No gross or microscopic pathology could be attributed to this diet. No metal toxicity was evident and photosensitization did not occur.

WILLSTÄTTER and Fisher, in 1915 and 1930, when they published their climatic researches on chlorophyll, stimulated the interest of Emil Bürgi, a Swiss (1), who in 1916 advocated the use of chlorophyll and its derivatives as therapeutic agents. Bürgi's enthusiasm was predicated upon the structural resemblance between erythrocyte hemin and the green pigment from the chloroplasts as disclosed by Willstätter and Fisher. However, in this suggestion of therapeutic value, Bürgi's thought that chlorophyll might be a hemopoetic agent was not original, for Verdeil (2) had suggested this use almost a century before. Furthermore, even before Willstätter and Fisher, the relationship between hemin and chlorophyll was suspected by Hoppe-Seyler (3), who had separated a red porphyrin from chlorophyll which was similar in many respects to one obtainable from hemin. Nevertheless, interest in chlorophyll or its derivatives as therapeutic agents lagged until the enthusiasm of Bürgi launched numerous scientific investigations which followed the marketing by his associates of chlorophyll products: both as chlorophyll itself, and as water-soluble derivatives of chlorophyll. The usefulness of these was a controversial issue almost from the start; careful investigators both supported and denied the suggested values. Lately chlorophyll, and especially the water-soluble derivatives of chlorophyll have become increasingly of interest, especially to stimulate tissue healing (4), and as deodorants, though their original suggested use as hemopoetic agents no longer persist.

Bürgi, Gordonoff, Grigoriew, Patek (5), and others, who were the initiators, employed "pure" chlorophyll, as well as various chlorophyll fractions in their studies, terming the products: sodium chlorophyllin, sodium and potassium chlorophyllin, "Nachlorophyllate," pheophytin and chlorin. That none of these were likely pure products, can be conceded in view of the difficulties presently encountered in preparing pure materials of this nature. The names used by Bürgi and others to describe the products employed were no doubt loosely applied. Actually little concern seems to have been given to their true identity, probably because it was impractical to prepare pure products, and even impractical to ascertain the identity of the fractions in the available marketed materials. This situation continues today, for we find that the extensively used metallo-complex known commercially as potassium sodium copper chlorophyllin, is in reality a mixture, and the term will be used with that meaning in mind in this paper. Examination of a typical production lot of this complex by Pickel, Scanlan, and Heggie (6) is illustrative of the complexity of the commercially marketed product (Table I). This is apparently typical of the acceptable and generally marketed, potassium sodium copper chlorophyllin.

TABLE I.—ANALYSIS OF LOT 89

	Per cent
Ash	32.33
Nitrogen (Kjeldahl)	3.88
Sodium	0.16
Potassium	14.49
Copper (total)	4.10
Copper (ionic at *p*H 3)	0.24
Phytol	None
Yellow Fraction	0.2
Isochlorin e_4	12.
Chlorin e_6[a]	36.
Assay (N. N. R.)	78.5
*p*H 1% solution	9.7

[a] Now known to be a mixture of chlorin e_6 and rhodin g_7.

Bürgi and others, as a result of their investigations in regard to therapeutic usefulness, published a considerable amount of data on various aspects of chlorophyll and its degradation prod-

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Supported by a research grant of the American Chicle Company.
Owing to the unusual length of this article, part of the cost of publication was borne by the author.

ucts. Some observations as to safety or toxicity naturally followed, but in these studies the therapeutic doses employed did not lend themselves to the more drastic approach which is followed today to establish safety or a tolerance level. A conclusion of safety within the realm of the necessities of the time was largely *a priori* drawn, likely by reason of chlorophyll's natural origin and on the basis of man's constant ingestion of chlorophyll in his diet. This conclusion was supported by a limited amount of animal experimentation at a toxic level. With the resurgence of interest in chlorophyll about 1940, especially regarding the water-soluble fractions and particularly the copper complex, Smith (7) investigated the probable safety of this metallic complex derivative of saponified and substituted chlorophyll, which he described as "sodium copper chlorophyllin." Employing 0.2 and 2 per cent aqueous solutions, Smith administered the material orally, intravenously, and intraperitoneally, for periods up to five days, to rabbits, and up to eight days to humans. He observed no ill effects in any of the subjects. However, these administrations were over a short period of time and do not furnish an answer relative to its chronic toxicity. Though Smith concluded from his experimental work, that there were no toxic effects, Corwin (8) postulated that the copper of this chlorphyll derivative might be available, thereby capable of causing liver damage, if ingested in sufficient quantity. Furthermore, Corwin concluded that a high intake of any chlorophyll derivative, especially those of simple form, might be inadvisable and extremely dangerious if effective amounts reached the blood stream. He concluded however, that such absorption is apparently not likely inasmuch as there is no evidence of such an effect among the population at large.

Zirm and Kilches (9), using what they describe as a water-soluble C^{14} labeled sodium magnesium chlorophyllin, found that it was not transported across the gastrointestinal membranes. There was no indication of its presence in the blood or of it being deposited in the tissues. White mice were employed and 10 mg. was administered orally. Excretion in the feces persisted for eight days, after the single dose.

An extensive review of the literature on chlorophyll has been made by Eddy (10). Reference to this will furnish additional information upon the history and use of these materials.

The present internal human uses of potassium sodium copper chlorophyllin are as a breath deodorant, which involves the ingestion of approximately 4 mg. per lozenge, and as a systemic deodorant, employing tablets containing 16 mg. As the compound contains not more than 6 per cent of copper, these doses would contribute a maximum of 0.24 mg. and 0.96 mg. respectively, of total copper, of which 0.012 mg. and 0.048 mg. is in ionic form at a *p*H of 3.

There is no general unanimity of opinion that the population at large requires a source of copper supplementary to the average diet, which is estimated by Darby (11) to contain 2–4 mg. per day, though a daily intake of 2 mg. is viewed as being the minimum required. That copper is a necessary element in the daily human diet is accepted, though the role it plays in nutrition is not understood (12). However, in animals, experimental anemia may be developed even in the presence of an adequate iron intake when copper is absent (13). Many common foods furnish rather considerable amounts of copper (14), and in some areas the copper intake in foods is increased by the practice of enhancing the color of canned green vegetables by "coppering," or by employing brass vessels for the purpose of cooking. Drummond (15) concluded that 50–180 mg. of copper per Kg. of food, may be present without harm; Long (16), that it is nontoxic when combined with the chlorophyll of green vegetables; Waltner (17) that 0.1 per cent in the diet has no effect upon rats; Huber (18) that 10 mg./Kg. daily to guinea pigs for seventy two days has no effect; Kiyooka (19) that 76 mg. daily to man is without effect. The intake of copper by humans may, therefore, in most instances be considerable and the amount that may be ingested by reason of the use of potassium sodium copper chlorophyllin may play no significant part. Especially may this be true if only the ionizable copper is available to the organism.

However, toxic effects follow a high intake of copper, over prolonged periods: Waltner states that 2 per cent in the diet of rats results in death in four to five days. Kiyooka that 750 mg. daily to man is toxic. Individual large doses are not likely to be harmful if the subject is physiologically capable of emesis, for such large doses are irritating to the stomach, and vomiting follows their ingestion.

The likelihood that copper will exhibit a toxic effect when it is continuously ingested is therefore governed by the level of intake and the availability of the ingested copper for transfer across the gastrointestinal membrane. However, administration of copper over a prolonged period, even at a low concentration, if it is available for absorption, results in increased copper storage especially in the liver and spleen (20).

The suggestion by Corwin that a high intake of

chlorophyll derivatives might result in photosensitization by reason of circulating porphyrins is also of interest. Chlorophyll itself, when freshly released, by direct contact with plants upon areas of the skin not normally exposed to light, is alleged to result in photosensitization. Furthermore, photosensitization is known to follow the administration of hemoglobin derivatives (hematoporphyrin), and some dyes, which effects have been employed as therapeutic measures. Photosensitization is not uncommon, as contact with parsnip, bergamot oil, or some fluorescent dyes (lip stick) may also result in contact photosensitization. Furthermore, cud chewers (ruminants) develop phylloerythrin, presumably from chlorophyll, in their digestive tract, a porphyrin known to be photosensitizing. This is normally excreted in the bile, but if excretion is prevented, photosensitization may follow. Indeed phylloerythrin is found in human feces and an increase in the amount present appears to follow an increase of chlorophyll intake. Furthermore, chlorophyll urinary porphyrin appears to be isomeric with blood porphyrin. Rothemund in his extensive review on chlorophyll outlines the possibilities of photosensitization (21).

Safety studies on potassium sodium copper chlorophyllin, employing the long-term study techniques designed in the manner of present-day practice, do not appear in the literature. The lack of this information and the presently important aspect of water-soluble chlorophyll products, resulted in the initiation of studies employing mice, rats, and guinea pigs as test animals. Because of the thought that the copper content of "chlorophyllin" might induce a toxic response, studies were also initiated using copper sulfate and copper gluconate as the copper salts for comparison of effects. The copper of these copper salts is 100 per cent ionizable, whereas the copper of potassium sodium copper chlorophyllin is about 5 per cent ionizable, fully 95 per cent being bound in a chelate position.

In designing the studies, consideration was given to many viewpoints, i. e., will these modified chlorophylls, when consumed at high level, influence growth, mating, or the normality of the tissues; will this "chlorophyllin" induce a high porphyrin content of the blood resulting in the possibility of photosensitization; will the content of 4 to 5 per cent of total copper in the "chlorophyllin" be toxic; will the copper interfere with the availability and use of vitamin C, which is known to deteriorate rapidly in the presence of copper.

The material commercially available is termed potassium sodium copper chlorophyllin, and it will be referred to as such, though it is now recognized that pure chlorophyllins do not comprise a major part of the commercial product; and for brevity in some instances the term "chlorophyllin" in quotation marks will be employed. In marketing it is also described as a "water-soluble chlorophyll" though there are many such soluble derivatives of chlorophyll, and the potassium sodium copper chlorophyllin complex is probably a colloid in polyelectrolyte dispersion (22).

ACUTE TOXICITY OF POTASSIUM SODIUM COPPER CHLOROPHYLLIN

In the acute toxicity tests Swiss mice, Taconic Farms[1] strain, and Sprague-Dawley[2] rats were used. The mice weighed between 18 and 24 Gm., the rats 180–240 Gm. To the mice the chlorophyllin was administered orally, in 15% aqueous solution by stomach tube and by intraperitoneal route. The rats received a diet containing 15% of the "chlorophyllin."

The LD_{50}, oral, for male mice was found to be 7.0 Gm. per Kg.; the approximate LD_0 being 5.0 Gm. per Kg. and the LD_{100}, 12 Gm. per Kg. Observations were made over a period of seven days, but no change in the survival rate occurred after seventy two hours. The LD_{50}, intraperitoneal, for mice was 0.19 Gm. per Kg.; the approximate LD_0 being 0.13 Gm. and the LD_{100} 0.32 Gm. All solutions were adjusted to a *p*H of 7.6 before administering, by the addition of the hydrochloric acid. When water-soluble chlorophyll is administered by the intraperitoneal or intravenous routes, its high tinctorial power and colloidal nature results in all the tissues being tinted green and concentration in the RES. This coloration is noticeable in the ears and eyes of albino animals.

Five male and four female young adult rats were fed a diet comprising 15% potassium sodium copper chlorophyllin and 85% Rockland rat meal. The animals were maintained in individual cages for a period of ten days, during which no deaths occurred. The males ingested 10.2 Gm. of the "chlorophyllin," and the females who consumed more food, had an intake of 13.4 Gm. Some loss in weight occurred in both sexes, a greater loss among the males, due to refusal of the food mixture. The over-all intake during the ten days averaged above 50 Gm. "chlorophyllin" per Kg. of rat. During a ten-day period the LD_0 for rats was therefore above 50 Gm. per Kg.

LIFE CYCLE TOXICITY STUDY (CHRONIC TOXICITY) ON RATS OF POTASSIUM SODIUM COPPER CHLOROPHYLLIN

Weanling rats of the Sprague-Dawley strain were housed individually to prevent coprophagy and in a manner whereby an accurate record of food and water intake of each animal would be assured. Two hundred animals were included in this study, forty in each of the feeding levels, namely 0.1%, 1%, and 3% of the "chlorophyllin" in the diet and a like

[1] Taconic Farms, Inc. Germantown, N. Y.
[2] Sprague-Dawley Company, Madison, Wis.

group upon the basic diet alone, which was Rockland rat diet in meal form, an adequate diet for rats consisting of required food elements.

In order to hold a reasonably consistent ratio of "chlorophyllin" intake per gram of animal weight over the wide life cycle weight range of the test animals, a moving percentage in the diet was maintained. During the first fourteen days on test when food intake is highest per gram of animal weight, 25% of the stated concentrations were fed; during the second fourteen days, 50% of the stated concentrations were fed; thereafter for the balance of the study the 0.1, 1.0, and 3.0% concentration in the diets were maintained.

The weight of each animal was determined weekly as well as the amount of food and water consumed. Animals were individually inspected no less than three times each week. Thus, there were four groups of animals, each group comprised twenty males and twenty females, and in each group a littermate of the same sex.

Growth (Gain in Weight).—During the first twenty months, the animals were on test, male animals were receiving up to 3% of "chlorophyllin" in the diet (roughly 3 Gm. "chlorophyllin" per Kg. of animal) and increased steadily and constantly in weight. An average gain in weight of 470 Gm. occurred among the control animals, and of 494 Gm. among the animals on the diet containing 3% "chlorophyllin" (Fig. 1). Female animals likewise gained in weight constantly. An average gain of 278 Gm. occurred among the control animals and 255 Gm. among those receiving 3% of the test material in the diet (Fig. 2). After this period of twenty months, terminal sacrifice, death due to age, and emaciation due to old age affected all groups so that the average total body weight and gain in weight show greater fluctuations.

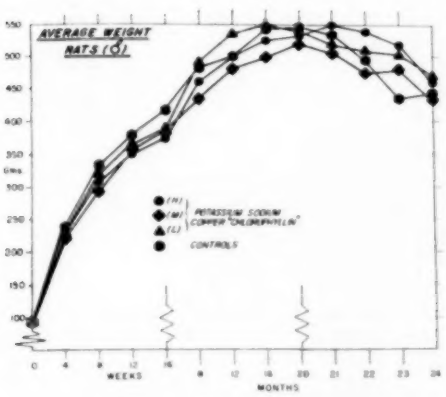

Fig. 1

The growth of animals on the other levels of intake, i. e., 0.1% and 1.0% was comparable. The gain in weight of the several groups during their growth periods as compared with control animals was not significantly different. The increase in weight, the standard error of the average weights and the number of animals in each group at specified

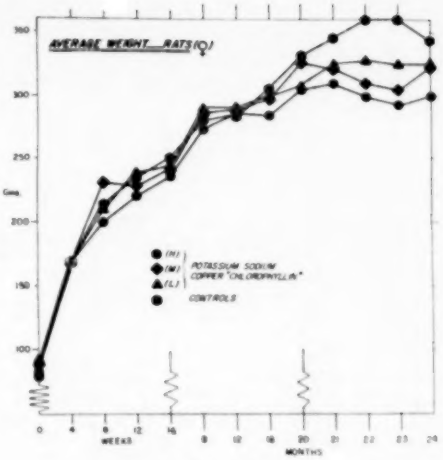

Fig. 2

periods during the study show no significant differences between the groups on the several levels of "chlorophyllin" and the control group, within the sexes (Table II). As the study neared termination, after the ninety-ninth week, the variations in weight within groups was larger as evidenced by the greater standard error (Table II), emaciation of old age, especially among the males, resulted in greater weight variations, and individual loss of weight.

Food Usage and Material Intake.—The net food intake over a ninety-three week period averaged within 3% for all groups, an average net daily food use of approximately 20 Gm. for males and 16 Gm. for females. During this period the females on the high (3%) intake of "chlorophyllin" consumed an amount greater than their own body weight (Table III). Male animals on the average consumed an amount equal to about four fifths their body weight.

The efficiency of food use expressed as grams of weight gain per gram of food consumed, was calculated for the periods terminating at the eighth and twelfth weeks. This usage did not differ materially between those animals on the control diet and those receiving "chlorophyllin" in their diets (Table IV).

Mating.—Five males and five females from each of the feeding levels were mated. The respective male of a specified level was mated with a female of the same level, allowing a one week residence of the male. Not all pairs conceived. The control animals whelped an average of 7.2 pups; the test groups 6.5–9 pups. From the control pups an average of 5.2 were raised to maturity; from the test groups, 4.5–6.2 pups.

Mortality.—The survival of animals was satisfactory: 30% being lost from the control group, 22% and 18% from the 3% and 0.1% diet groups, respectively (Fig. 3), the majority of deaths occurring during the last few weeks.

Blood and Urine Examination.—Routine hematologic and urine examinations were made several times during the twenty months, as well as nonprotein nitrogen determinations upon the blood. The factors determined were within normal limits.

TABLE II.—AVERAGE BODY WEIGHT OF RATS RECEIVING POTASSIUM SODIUM COPPER CHLOROPHYLLIN IN DIET

		0 week	4 weeks	8 weeks	12 weeks[b]	6 months	1 year	99 weeks[c]
Females								
Controls		52 ± 3.0 Gm.	168 ± 3.5 Gm.	212 ± 3.6 Gm.	235 ± 3.5 Gm.	261 ± 4.2 Gm.	286 ± 5.8 Gm.	359 ± 33.6 Gm.
	N	20	20	20	16	15	15	11
Potassium sodium copper chlorophyllin								
0.1% in diet		52 ± 1.6[a]	168 ± 3.4	210 ± 9.3	237 ± 4.6	267 ± 4.7	288 ± 4.8	328 ± [illegible] 4
	N	20	20	20	15	15	15	[illegible]
1.0% in diet		50 ± 2.4	167 ± 3.8	229 ± 5.1	227 ± 3.7	258 ± 4.5	290 ± 4.2	308 ± 43.4
	N	22	22	22	18	18	18	11
3.0% in diet		49 ± 3.0	165 ± 3.3	202 ± 3.6	220 ± 4.1	248 ± 4.7	287 ± 8.0	299 ± 28.9
	N	20	20	20	16	16	16	9
Males								
Controls		53 ± 3.4	223 ± 21.0	335 ± 4.6	382 ± 27.2	422 ± 24.1	500 ± 15.4	440 ± 92.1
	N	20	20	20	16	15	15	8
Potassium sodium copper chlorophyllin								
0.1% in diet		54 ± 3.6	228 ± 21.1	320 ± 20.9	366 ± 5.8	421 ± 6.3	533 ± 10.0	519 ± 60.6
	N	20	20	20	16	16	16	8
1.0% in diet		54 ± 2.6	227 ± 5.5	294 ± 7.3	360 ± 5.6	403 ± 10.8	481 ± 15.9	461 ± 8.8
	N	18	18	18	14	14	14	7
3.0% in diet		54 ± 3.7	223 ± 7.4	311 ± 6.0	352 ± 8.0	393 ± 10.0	500 ± 15.8	495 ± 15.5
	N	20	20	20	16	16	16	7

[a] Standard Error = $\sqrt{\Sigma d^2/N(N-1)}$.
[b] Depleted by autopsy at 10th week.
[c] Depleted by autopsy at 52nd week.

TABLE III.—AVERAGE AMOUNT OF FOOD AND OF "CHLOROPHYLLIN" IN GRAMS CONSUMED PER RAT IN 93 WEEKS

Chlorophyllin in Diet	Food Consumed		"Chlorophyllin" Consumed	
	Females	Males	Females	Males
Control	10,760	12,810	None	None
Chlorophyllin				
0.1%	10,730	13,110	10.6	12.5
1.0%	10,680	13,050	105.5	129.1
2.0%	11,240	13,610	333.2	403.9

TABLE IV.—FOOD EFFICIENCY AND GAIN IN WEIGHT

	Gain in Weight, Gm.				Food Efficiency, Gm. Gained per Gm. Food Consumed	
	8 weeks	12 weeks	26 weeks	52 weeks[a]	8 weeks	12 weeks
Males						
Controls	238	285	326	404	0.23	0.17
Potassium sodium copper chlorophyllin						
0.1% in diet	224	270	325	437	0.23	0.18
1.0% in diet	202	268	310	388	0.21	0.17
3.0% in diet	218	260	300	407	0.20	0.15
Controls	229	301	359	378	0.24	0.20
Copper sulfate						
(530 p. p. m. Cu)	202	286	353	359	0.25	0.22
(1,600 p. p. m. Cu)	176	209	241	254	0.23	0.17
Copper gluconate						
(1,600 p. p. m. Cu)	197	252	193	144	0.24	0.19
Females						
Controls	129	152	178	204	0.15	0.12
Potassium sodium copper chlorophyllin						
0.1% in diet	119	146	176	197	0.15	0.12
1.0% in diet	139	137	168	200	0.16	0.11
3.0% in diet	127	137	164	204	0.15	0.10
Controls	131	153	188	192	0.15	0.12
Copper sulfate						
(530 p. p. m. Cu)	140	165	204	194	0.19	0.14
(1,600 p. p. m. Cu)	125	151	147	184	0.17	0.13
Copper gluconate						
(1,600 p. p. m. Cu)	125	160	129	107	0.16	0.13

[a] For copper sulfate and copper gluconate, 35 weeks.

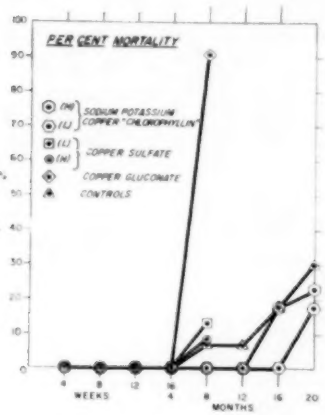

Fig. 3

Oxygen Carrying Capacity.—Hemoglobin was also determined by the Van Slyke procedure: oxygenating by gently rotating a thin layer of the blood sample, followed by gasometric determination of the oxygen. These results when compared with those obtained by the iron and acid hematin methods, upon samples removed at the third, fifth, and twelfth week disclosed no effect by the "chlorophyllin" upon the oxygen carrying capacity of the hemoglobin.

Gross Pathology.—Animals sacrificed at the tenth, fifty-second, and approximately the hundred and fourth week, exhibited the usual expected findings upon autopsy. These findings were distributed among the control as well as the test groups of animals. They comprised, in those animals sacrificed at two years, ventricular edema; consolidated areas in the lung; occasional liver tumor and occasional cystic areas; retention cysts and minor congestion of the kidneys; several pituitary tumors; hyperplasia of lymphoid tissue of the small intestine; small reproductive organs; all of these being findings consistent with the age and strain of animals.

Organ Weights.—At the time of sacrifice and autopsy of animals at the fifty-second and one hundred and fourth week, the weight of the principal organs was determined. These weights were calculated to the gram weight of the tissue per 100 Gm. weight of the test animal, and are reported herewith (Table V). There were no significant differences.

Plasma "Chlorophyllin" and Copper.—When potassium sodium copper chlorophyllin is fed to rats at a sufficient concentration in the diet, it is transmitted across the gastrointestinal membrane and appears in the plasma. Under such conditions the plasma is tinted and contains determinable amounts, when the diets of the animals contain more than 0.1% of the "chlorophyllin" for at least several days. The amount of "chlorophyllin" and of copper is variable and is governed by the concentration of the "chlorophyllin" in the food which is ingested (Table VI). For the purpose of determination, blood is collected into heparin by cannulation of an external carotid artery, and then gently centrifuged. The centrifuged plasma is separated, clarified by the addition of nine parts of ethyl alcohol which has been previously adjusted to a *p*H of 9 by the addition of sodium hydroxide. The centrifuged clear alcohol dilution of the plasma is then read at 400 mμ, against a similarly treated plasma from

TABLE V.—AVERAGE WEIGHT OF TISSUES, GRAMS PER 100 GM. OF BODY WEIGHT

	N	Heart	Lungs	Liver	Spleen	Kidneys	Uterus (Seminal Vesicles)	Ovaries (Testes)	Stomach	Brain	Approx. Weeks on Test
					Females						
Controls	6	0.384	0.554	3.902	0.203	0.816	0.256	0.040	0.634	0.614	104
Potassium sodium copper chlorophyllin											
0.1% in diet	10	0.367	0.555	3.559	0.240	0.799	0.285	0.078	0.645	0.616	104
1.0% in diet	9	0.371	0.590	4.375	0.213	0.855	0.263	0.040	0.708	0.615	104
3.0% in diet	7	0.359	0.670	3.632	0.232	0.953	0.346	0.042	0.758	0.712	104
					Males						
Controls	4	0.358	0.526	3.564	0.208	0.857	0.227	0.737	0.661	0.488	104
Potassium sodium copper chlorophyllin											
0.1% in diet	6	0.415	0.532	3.946	0.179	0.872	0.189	0.688	0.686	0.584	104
1.0% in diet	5	0.366	0.704	4.419	0.177	1.190	0.193	0.537	0.725	0.506	104
3.0% in diet	4	0.347	0.404	4.015	0.176	0.921	0.225	0.699	0.770	0.506	104
					Females						
Controls	9	0.317	0.500	3.214	0.203	0.717	0.274	0.038	0.615	0.656	42
Copper sulfate (530 p. p. m. Cu)	15	0.295	0.553	3.250	0.182	0.714	0.212	0.037	0.628	0.630	42
(1,600 p. p. m. Cu)	10	0.301	0.564	3.778	0.209	0.799	0.179	0.040	0.821	0.684	42
Copper gluconate (1,600 p. p. m. Cu)	4	0.329	0.554	4.825	0.255	0.894	0.078	0.024	1.127	0.824	42
					Males						
Controls	8	0.268	0.495	3.586	0.169	0.798	0.827	0.350	0.518	0.424	42
Copper sulfate (530 p. p. m. Cu)	12	0.282	0.487	3.674	0.189	0.792	0.666	0.357	0.585	0.423	42
(1,600 p. p. m. Cu)	6	0.301	0.488	4.072	0.198	0.889	0.839	0.405	0.686	0.505	42
Copper gluconate (1,600 p. p. m. Cu)	2	0.419	0.967	3.940	0.198	1.052	0.760	0.157	1.227	1.136[a]	42
					Females						
Controls	4	0.336	0.770	3.524	0.188	0.753	0.230	0.039	0.645	0.668	33
Copper sulfate (1,600 p. p. m. Cu)	4	0.333	0.569	3.767	0.185	0.670	0.135[b]	0.024[b]	0.795	0.669	33
Copper gluconate (1,600 p. p. m. Cu)	4	0.378	0.676	4.465	0.215	0.782	0.081	0.022	1.029	0.648	33
					Males						
Controls	4	0.301	0.713	3.556	0.173	0.777	0.923	0.359	0.531	0.479	33
Copper sulfate (1,600 p. p. m. Cu)	4	0.297	0.518	3.492	0.176	0.720	0.700	0.255	1.061	0.572	33
Copper gluconate (1,600 p. p. m. Cu)	4	0.328	0.553	3.963	0.205	0.891	1.008	0.286	1.013	0.664	33

[a] 1 animal only.
[b] 3 animals only.

control animals, in a Beckman model DU Spectrophotometer, using a slit width of 0.08 mm.

By trial it was found possible to recover 60% of "chlorophyllin" added to plasma obtained from control rats after this plasma with the added "chlorophyllin" was clarified in the manner described. A concentration of 25 μg./ml. was detectable and the recovery uniform over a range of 30 μg. to 150 μg./ml. (corrected). The separated precipitated plasma fractions are of a greenish hue, and the chlorophyllin is firmly bound therein. An assumption of course, enters into the conclusion, namely, that the chlorophyllin read in the plasma fraction has the same spectral characteristics as that added to plasma obtained from control animals.

Other methods of clarifying the plasma to make the readings possible were not as successful, inasmuch as the "chlorophyllin" in the plasma is readily precipitated at lower *p*H values.

The copper content of the plasma is not in agreement with that which should be present by reason of the "chlorophyllin" content. A copper content of 615 μg./100 ml. over and above that present normally in the plasma should be found when "chlorophyllin" is present to the extent of 116 μg./ml. The excess copper found was approximately 100 μg./100 ml. Upon the lower diet intake of "chlorophyllin" excess copper was not observed. Copper was determined by the method of Gubler *et al.*, (23).

Fecal "Chlorophyllin."—Though "chlorophyllin" as ingested is soluble in water, a 1% solution having a *p*H of 9.7, is precipitated upon the addition of acid, and redissolved in the presence of alkali. However, "chlorophyllin" excreted in the feces is no longer soluble either in water or in mild alkali. In fact the feces, though vividly green, are not completely extractable by organic solvents and it is only upon treatment with glacial acetic acid and repeated extraction with ether that an appreciable quantity of the "chlorophyllin" is removed in the separated ether fractions. The remaining feces are still distinctly green in color. These ether extractions were subsequently partitioned between 5, 10, 25, and 37% HCl according to the usual methods (24). The acid extractions are subsequently neutralized with sodium hydroxide-sodium acetate, re-extracted with ethyl ether, and the ether solution of the acid fractions then read spectrophotometrically in the ultraviolet and visible range on a Beckman model DU. A sample of 100 mg. of dried feces was extracted in this manner. A quantity of "chlorophyllin" was subjected to the same procedure and read in the same manner for comparison. The curves of transmittance in the ultraviolet range of the several "chlorophyllin" acid fractions obtained from feces did not differ materially from the curves resulting when "chlorophyllin" itself is treated in the same way (Fig. 4). The curves obtained in the visible range for the fecal "chlorophyllin" acid fractions as regards that portion of the "chlorophyllin" which is *not* extractable by acid show a shift in the maximum peaks of absorption from 520–540 mμ and 620–660 mμ (Fig. 5). The rest of the fecal acid fractions are comparable in the visible range with the direct "chlorophyllin" fractions.

The 5% acid fractions which would include both porphyrins and phylloerythrin, are fluorescent; the 10% acid fractions which might include phylloerythrin are nonfluorescent. However, there is no phylloerythrin absorption peak at 520 mμ, in either the 5% or 10% HCl fractions both of which extract phylloerythrin.

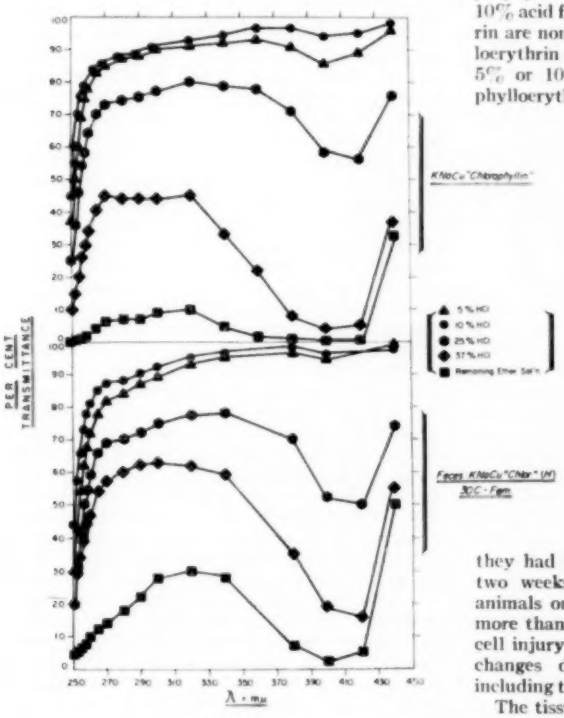

Fig. 4

Ingested "chlorophyllin" is excreted in the feces essentially in an insoluble form, probably occurring as a calcium complex which is difficult to cleave. Copper is present, apparently tightly bound to the chlorophyll moiety.

Tissue-Stored Copper and Iron.—An examination was made of the liver, kidneys, and spleen of animals sacrificed at the tenth, fifty-second, and one hundred and fourth week, for the copper and iron content, of the tissues. These findings will be discussed in the section *Tissue-Stored Copper and Iron* under that part referring to the administration of other copper compounds.

Histopathology.—The kidneys, liver, stomach, small intestine, and spleen of animals sacrificed after they had been in the study for a period of fifty-two weeks were examined. The livers of those animals on the high level (3%) intake showed no more than tinctorial changes without indication of cell injury. All other tissues disclosed only minor changes distributed among the several groups including the controls.

The tissues of those animals in the control group and the high level (3%), which were sacrificed at the

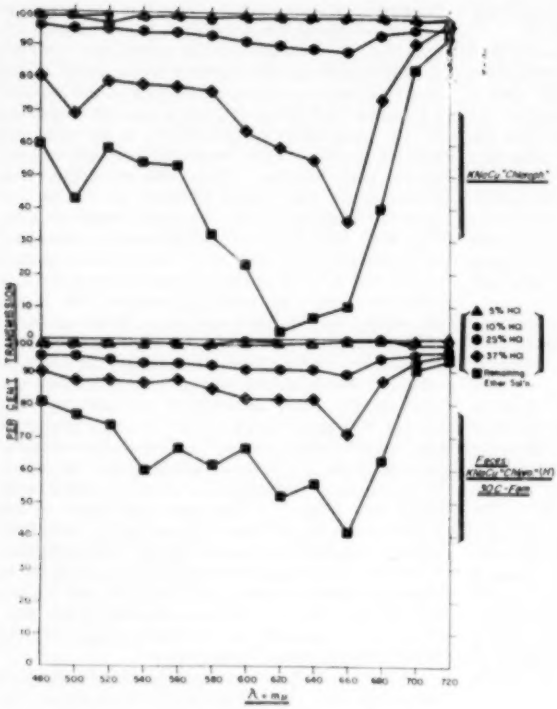

Fig. 5

TABLE VI.—"CHLOROPHYLLIN" AND COPPER CONTENT OF PLASMA FROM ADULT MALE RATS AFTER 62 DAYS ON THE SPECIFIED DIET

Data from Pooled Samples of Plasma from 3 Rats on Each Level

Diet	"Chlorophyllin," μg./ml.	Copper, μg./100 ml.
Control diet	None	189
"Chlorophyllin"		
0.1%	None	174
1.0%	58	196
3.0%	116	303

termination of the study (one hundred and four weeks) were examined with findings as follows: *Kidneys.*—All sections of the control and test animals showed interstitial scarring, tubular atrophy, dilated tubules filled with hyaline material and minor inflammatory changes. It appears that the changes are related to the age of the animals rather than to the product. *Livers.*—All livers were well within the normal limits. *Spleen.*—All spleens were within normal limits. *Adrenals.*—Changes of a cystic and old hemorrhagic nature in the cortex of two high level (3%) animals and a small adenoma in an animal of the same level were observed. *Testicles and Seminal Vesicles.*—These were normal in all animals. *Ovaries and Uterus.*—These showed a normal histology when compared with their controls. *Gastrointestinal Tract.*—Those sections taken from the upper stomach, including a portion of the esophagus, from the duodenum with a fragment of pancreas attached; and from the large intestines showed in both the control and test animals a normal histopathology especially with respect to the mucosa and submucosa where pathology if present would show itself most prominently. *Nerve Tissue (Sciatic).*—There is no evidence that the material given, either changes the nerve sheath histologically or gives rise to fibrotic or a chronic inflammatory state. *Heart.*—The test animals compared with the controls showed no particular change.

SUMMARY

Aside from minor adrenal cortical changes there was no evidence of adverse effects of the administered substance upon the organs examined. The cortical changes in themselves could well be associated with old age.

LIFE CYCLE TOXICITY STUDY (CHRONIC STUDY) ON RATS OF OTHER COPPER COMPOUNDS

Though potassium sodium copper "chlorophyllin" as marketed contains between 4 and 5% of copper, this copper appears to be rather firmly bound. About 0.25% of copper can be recovered as soluble copper when "chlorophyllin" is dissolved in water and the solution adjusted to a *p*H of 3, by the slow addition of hydrochloric acid, then filtered. Thus better than 90% of the copper appears to be firmly bound in the molecule from which it is not released by hydrochloric acid at a *p*H of 3. Under the same experimental conditions, both copper sulfate and copper gluconate yield all of their copper.

When 3% of the "chlorophyllin" is incorporated in the diet, correcting by the presently conventional used assay procedure for "chlorophyllin," this diet contains 1,600 p. p. m. of copper, a concentration of copper which, if administered as copper sulfate, is known to retard growth of the rat and result in metal toxicity. However, growth retardation was not observed among animals which received "chlorophyllin" in a preliminary study; this, together with the evidence that copper is not at least released appreciably *in vitro*, supported an assumption that the majority of the copper in potassium sodium copper chlorophyllin might not be available to the animal. However, to confirm this, a secondary study was initiated wherein weanling Sprague-Dawley rats were placed upon diets containing copper sulfate equivalent in copper content to the copper content of the 3% and 1% potassium sodium copper chlorophyllin diets. These levels corresponded to 1,600 p. p. m. and 530 p. p. m. respectively of copper as copper sulfate in the diets. A similar group of animals was placed upon a diet containing copper in the form of gluconate equivalent to 1,600 p.p.m. copper in the diet. These concentrations were moving concentrations, i. e.,

TABLE VII.—AVERAGE BODY WEIGHTS OF RATS RECEIVING COPPER SULFATE OR COPPER GLUCONATE IN THE DIET

		0 week	4th week	8th week	12th week	20th week	35th week
				Females			
Controls	N	73 ± 2.3 Gm.[a]	172 ± 3.2 Gm.	204 ± 4.0 Gm.	220 ± 3.9 Gm.	261 ± 4.5 Gm.	265 ± 4.3 Gm.
		25	24	24	24	24	24
Copper sulfate (anhyd.) 0.135% in diet		67 ± 3.3	154 ± 2.8	207 ± 3.5	232 ± 3.2	270 ± 3.5	260 ± 5.1
	N	25	25	25	25	25	25
0.406% in diet		73 ± 2.2	153 ± 3.4	198 ± 2.7	224 + 3.1	220 ± 4.2	257 ± 3.6
	N	25	25	25	25	24	20
Copper gluconate 1.147% in diet		75 ± 2.5	170 ± 2.9	200 ± 3.1	235 ± 4.1	204 ± 3.8	182 ± 11.7
	N	25	25	25	25	23	6[b]
				Males			
Controls	N	81 ± 2.3	218 ± 7.2	310 ± 6.2	382 ± 7.0	438 ± 17.3	459 ± 17.3
		23	23	23	23	23	22
Copper sulfate (anhyd.) 0.135% in diet		72 ± 3.4	194 ± 6.5	279 ± 1.3	358 ± 5.8	425 ± 10.7	431 ± 3.7
	N	25	25	25	25	24	23
0.406% in diet		71 ± 9.3	174 ± 5.7	247 ± 6.3	280 ± 9.1	282 ± 10.6	335 ± 9.5
	N	23	23	23	23	20	16
Copper gluconate 1.147% in diet		75 ± 2.7	198 ± 7.5	272 ± 7.6	327 ± 6.5	268 ± 8.3	219 ± 11.0
	N	22	22	22	22	15[b]	2

[a] Standard Error.
[b] Depleted due to high mortality.

25%, 50% and then 100% of the stated amount after twenty-eight days, as described under the "chlorophyllin" study. The several levels of the "chlorophyllin" and the other copper salts are keyed in the figures as: high (H)—1,600 p.p.m. Cu, middle (M)—530 p.p.m. Cu, and low (L)—53 p. p. m. Cu. A control group was maintained concurrently on the basic diet which, as in the previous study, was Rockland rat meal. Each group contained fifty animals, equally divided between the sexes.

Growth and other data accumulated from this study may be compared with similar data gathered in the life cycle study on potassium sodium copper chlorophyllin.

Growth (Weight Gain).—Animals on the high level of copper sulfate and those upon copper gluconate were adversely affected in growth (Table VII). This retardation became readily discernible at the twenty-sixth week, when the male control animals and the animals receiving 530 p. p. m. of copper as copper sulfate weighed at least 50% more than those animals upon the 1,600 p. p. m. copper intake, either as sulfate or as gluconate (Fig. 6). Animals upon 1,600 p. p. m. copper as potassium sodium copper chlorophyllin were not affected in regard to weight gain. The adverse effect of copper as gluconate upon growth was the most marked. However, the lower growth rate is not due entirely to a toxic effect, at least during the first twelve weeks, as the total intake of food was less and the gain in weight per gram of food consumed was similar for all groups (Table IV). The accentuated toxic effect of copper in this form was also discernible in the death rate of this group between the fourth and eighth month, during which time nearly 90% of the animals died (Fig. 3). Because it was feared all of the copper gluconate animals would be lost, four animals of the control group, the high level copper sulfate, and the copper gluconate groups were sacrificed shortly thereafter. The balance of the animals were continued in the study and all surviving animals of all groups were sacrificed at the fortieth to forty-fourth week.

Blood and Urine Examinations.—Routine hematologic and urine examinations were performed at intervals. All factors were within normal expected ranges, except blood nonprotein nitrogen levels. High NPN was noted in the males,

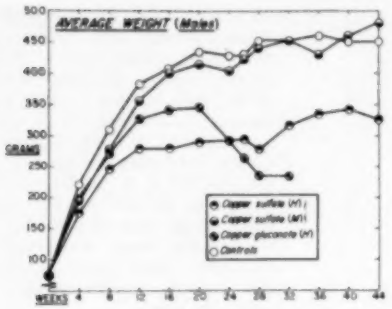

Fig. 6

being 83 mg. % for the animals receiving copper sulfate (1,600 p. p. m. Cu), and 109 mg. % for the copper gluconate (1,600 p. p. m. Cu) animals. The lower level copper sulfate male animals, and the female animals on all levels of copper were just above the expected range of 60–70 mg. % of non-protein nitrogen.

Oxygen Carrying Capacity.—Gasometric determinations of the oxygen carrying capacity of the blood compared satisfactorily with the hemoglobin value determined by the iron and acid hematin methods.

Organ Weights.—The average weight of the various organs per 100 Gm. weight of animal, were within the expected ranges as compared to the controls of the same age, except certain tissues. Animals receiving copper gluconate had hypertrophied uteri, ovaries, or seminal vesicles. The stomachs of female animals on the high level of copper sulfate and those of the females and males receiving the diet containing copper gluconate were enlarged (Table V).

Gross Pathology.—A number of animals were sacrificed between the thirtieth and thirty-fifth week to avoid the possible loss of all animals receiving copper gluconate. All surviving animals were sacrificed during the fortieth to forty-fourth week. Aside from findings that were distributed throughout all the groups and expected in the animals of the indicated age, the following were common to the test groups upon the higher intake of copper salts: Bronzed kidneys exhibiting sharp demarcation between the cortex and the medulla; bronzed or yellowish livers; hypertrophied ridges between the cardiac and peptic portions of the stomach, occasional ulcer, some blood; bloody mucous in the intestinal tract. The stomachs of some of those animals receiving copper gluconate were often flabby and distended. These observations were noted especially among the animals upon the high intake of copper sulfate and copper gluconate, which is in

TABLE VII.—COPPER CONTENT OF TISSUES OF RATS RECEIVING POTASSIUM SODIUM COPPER CHLOROPHYLLIN IN DIET, MG. Cu/100 GM. TISSUE (WET BASIS)

	Control Diet		Potassium Sodium Copper Chlorophyllin					
			0.1%		1.0%		3.0%	
	M	F	M	F	M	F	M	F
Liver								
10 weeks								
Av.	0.41	0.48	0.47	0.57	0.58	0.74	0.56	0.56
S.E.	0.04	0.08	0.026	0.09	0.035	0.065	0.06	0.08
N.	4	4	4	4	4	4	4	4
52 weeks								
Av.	0.78	1.09	1.46	1.14	0.81	2.43	1.06	2.14
S.E.	0.020	0.052	0.64	0.29	0.064	1.12	0.42	0.711
N.	3	3	3	3	3	3	3	3
104 weeks								
Av.	1.82	1.10	1.47	1.85	1.85	2.02	2.18	3.71
S.E.	0.58	0.152	0.304	0.251	0.504	0.51	0.61	1.28
N.	4	6	6	10	5	9	4	7
Kidney								
10 weeks								
Av.	1.07	1.72	1.47	1.52	1.58	1.57	1.48	1.65
S.E.	0.15	0.57	0.27	0.11	0.51	0.16	0.32	0.22
N.	4	4	4	4	4	4	4	4
52 weeks								
Av.	2.08	4.46	1.52	2.44	1.83	3.79	2.11	2.97
S.E.	0.17	2.20	0.27	0.55	0.364	0.847	0.015	0.11
N.	3	2	3	3	3	3	3	3
104 weeks								
Av.	3.45	2.25	2.03	2.55	2.35	3.19	2.48	3.22
S.E.	0.91	0.23	0.709	0.19	0.727	0.393	0.63	0.416
N.	4	6	5	10	5	9	4	6
Spleen								
10 weeks								
Av.	0.96	1.59	0.52	0.46	0.40	0.72	0.68	0.52
S.E.	0.42	0.05	0.30	0.03	0.48	0.38	0.11	0.18
N.	2	2	2	2	2	2	2	2
52 weeks								
Av.	1.83	4.00	2.92	3.26	3.05	3.46	2.36	3.61
S.E.	0.58	1.02	1.45	1.02	1.36	0.817	1.03	1.89
N.	2	3	3	3	3	3	2	3
104 weeks								
Av.	3.38	6.96	3.34	1.92	2.75	2.34	3.01	2.96
S.E.	1.44	2.22	0.408	0.396	0.513	0.386	0.775	0.685
N.	4	6	6	10	5	9	4	7

TABLE IX.—IRON CONTENT OF TISSUES OF RATS RECEIVING POTASSIUM SODIUM COPPER CHLOROPHYLLIN IN DIET, MG. Fe/100 GM. TISSUE (WET BASIS)

	Control Diet		Potassium Sodium Copper Chlorophyllin					
			0.1%		1.0%		3.0%	
	M	F	M	F	M	F	M	F
Liver								
10 weeks								
Av.	2.36	2.50	2.25	3.56	1.57	2.26	3.22	2.09
S.E.	0.83	0.27	0.12	0.43	0.53	0.37	0.59	0.82
N.	2	2	2	2	2	2	2	2
52 weeks								
Av.	2.64	7.79	2.14	11.20	5.15	7.83	3.17	8.76
S.E.	0.458	0.341	0.258	1.92	2.50	2.35	0.441	0.452
N.	3	3	3	3	3	3	3	3
104 weeks								
Av.	17.7	24.7	16.6	27.0	15.7	24.9	18.0	31.3
S.E.	1.9	8.44	2.32	3.67	2.94	3.90	2.85	6.45
N.	4	6	6	10	5	9	4	7
Kidney								
52 weeks								
Av.	7.45	11.22	10.82	16.76	13.86	19.69	10.73	16.21
S.E.	1.32	1.95	2.40	3.59	2.40	1.01	2.41	2.67
N.	3	2	3	3	3	3	3	3
104 weeks								
Av.	19.9	32.4	24.7	25.6	17.4	31.1	23.5	28.3
S.E.	1.4	7.7	1.88	2.12	4.0	2.83	2.61	4.88
N.	4	6	6	10	5	9	4	6
Spleen								
104 weeks								
Av.	219.0	229.4	162.6	190.9	160.5	206.8	235.4	279.6
S.E.	24.6	32.7	30.4	17.3	27.5	27.9	13.0	41.2
N.	4	6	6	10	5	9	4	7

contrast to the relatively normal findings observed among animals receiving potassium sodium copper "chlorophyllin" contributing the same concentration of copper.

Histopathology.—Histopathological studies were performed on the organs of test animals receiving the high level of copper sulfate and gluconate, which were sacrificed after thirty to thirty-five weeks and also on the liver, kidney, and testes of animals receiving the lower level of copper sulfate sacrificed after forty to forty-four weeks. The results of these studies indicated the following organs to be normal in all the test animals as well as the controls: spleen, adrenals, small intestines, large intestines, stomach, and sciatic nerve. Kidney sections of those on the high level of copper sulfate and gluconate, did, however, show minor changes which did not correlate well enough throughout the animals to draw any definite conclusion. A study of liver sections of the animals receiving these copper salts revealed well defined abnormalities of a toxic nature in both the males and the females in that their icteric pigmentation was increased and cytoplasmic staining properties were abnormal.

Varying degrees of testicular degeneration were noted in both the high and low levels of the copper sulfate animals; the ovaries of the females on this product were not noticeably affected to any degree. The kidneys, liver, and testes of all the control animals were found to be normal.

Tissue-Stored Copper and Iron.—The liver, kidneys, and some spleens of animals from all groups were examined as to their total copper and iron content (Tables VIII to X).

Liver copper averaged less than 2 mg. per 100 Gm. of tissue in those animals receiving the control diet, and the two diets containing 0.1% and 1.0% of potassium sodium copper chlorophyllin. Those animals receiving 3% of "chlorophyllin" had a somewhat higher liver copper content which was non-significant and which concentration was noted only after the animals had been on the diet for a period of two years. Animals receiving 530 p. p. m. of copper in the form of sulfate, stored in forty weeks, 12 mg. to 32 mg. per 100 Gm. of liver, i. e., a storage of more copper than the high level "chlorophyllin" animals exhibited in a period of two years. Furthermore, the liver of animals which received 1,600 p. p. m. of copper as copper sulfate had deposited in them 38–46 mg. of copper per 100 Gm of tissue (Fig. 7). Comparing these figures with the copper

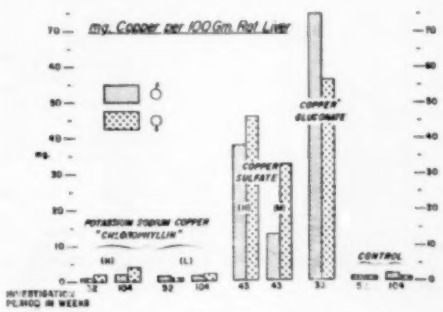

Fig. 7

found in the liver of animals receiving 1,600 p. p. m. of copper as gluconate, there is even a more marked difference, i. e., the liver of these latter animals

TABLE X.—COPPER AND IRON CONTENT OF TISSUES OF R. RECEIVING COPPER SULFATE OR COPPER GLUCONATE IN DIET, Mg. Fe or Cu/100 Gm. TISSUE (WET BASIS)

	Control Diet		Copper Sulfate, Cu 530 p. p. m.		Copper Sulfate, Cu 1,600 p. p. m.		Copper Gluconate Cu 1,600 p. p. m.	
	M	F	M	F	M	F	M	F
Copper Content								
Liver								
Av.	1.16	1.78	12.47	32.36	38.28	45.77	75.1	56.6
S.E.	0.31	0.39	2.52	14.6	13.85	5.18	12.07	6.10
N.	6	6	6	5	6	6	6	6
Kidney								
Av.	2.48	3.53	3.49	6.91	15.83	12.11	59.57	54.1
S.E.	0.20	0.33	0.54	0.48	6.21	4.80	14.75	21.5
N.	6	6	6	6	6	6	5	5
Spleen								
Av.	3.34	4.83	5.63	5.12	13.91	6.07	12.39	13.77
S.E.	0.63	0.33	1.5	1.3	7.50	1.72	3.9	3.29
N.	6	6	6	6	6	6	6	6
Iron Content								
Liver								
Av.	9.7	14.74	18.0	16.5	14.1	10.5	5.9	8.5
S.E.	2.5	4.0	9.6	1.6	6.3	5.2	2.3	5.0
N.	6	6	6	5	6	6	6	6
Kidney								
Av.	16.4	17.44	12.6	15.0	11.8	14.8	10.6	9.0
S.E.	1.4	1.74	1.97	0.98	1.7	1.5	1.2	2.0
N.	6	6	6	6	6	6	5	5
Spleen								
Av.	128.1	191.7	120.3	292.1	108.9	148.7	49.7	86.1
S.E.	18.9	37.3	13.6	12.4	18.7	41.7	11.4	41.7
N.	6	6	6	6	6	6	6	6

stored 56–75 mg. of copper per 100 Gm. of tissue, a concentration nearly twice that produced by administering an equivalent amount of copper sulfate and attained in less time, i. e., in approximately forty weeks *vs.* thirty weeks.

Liver storage of copper appears not to be significantly different between the male and female inasmuch as the high averages, as they occur, invert between the sexes.

The high storage of copper by the copper gluconate animals correlates with the high death rate of these animals and the high blood nonprotein nitrogen. There is also marked evidence among these animals of unfavorable gross pathology and histopathology.

In the kidneys (Fig. 8) and the spleen (Fig. 9)

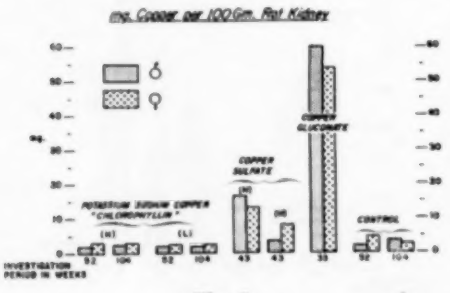

Fig. 8

animals receiving in the diet as high as 3% "chlorophyllin" (1,600 p. p. m. Cu) stored no more copper than animals on the control diet. There is, however, a somewhat higher storage of copper in the tissues

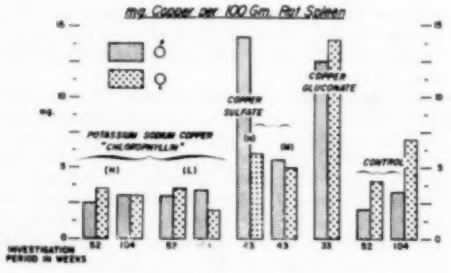

Fig. 9

of those animals receiving copper sulfate (530 p. p. m. Cu), which is accentuated among the animals receiving the high level of copper sulfate (1,600 p. p. m. Cu); furthermore, in the kidneys of those animals receiving copper gluconate (1,600 p. p. m Cu), there was deposited nearly twenty times the quantity of copper found in the tissues of the control animals.

Concurrently with the examination of the tissues for their copper content, a determination of the iron content was made. Iron storage does not differ between the controls and those animals receiving "chlorophyllin" in the diet, though iron storage in the liver appears to be somewhat greater in the female (Fig. 10). However, when there is a high storage of copper as in the instance of the animals receiving copper sulfate and copper gluconate, this appears to prevent in part or depress the storage of iron (Table X).

A similar situation exists in the kidneys, and the females again appear to store generally a greater

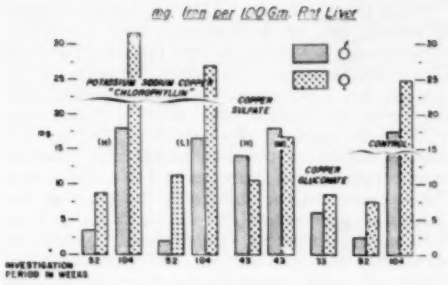

Fig. 10

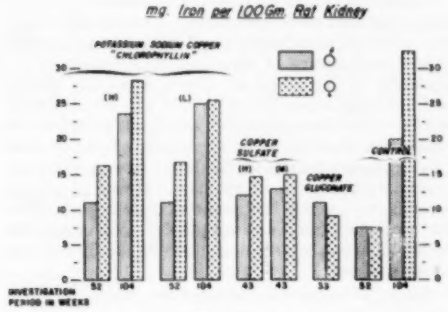

Fig. 11

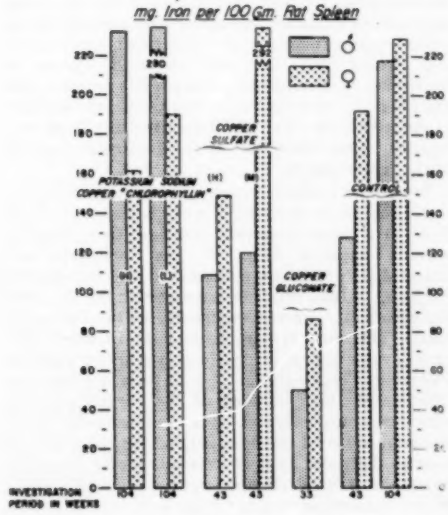

Fig. 12

quantity of iron (Fig. 11). Storage of iron in the female spleen is greater than in the male and is apparently markedly depressed by the storage of excess copper (Fig. 12).

An increased iron storage in all tissues is strikingly evident in animals of greater age.

Tissues were dry ashed at 525°, the copper determined by the diethylthiocarbamate procedure, and the iron by a modified *o*-phenanthroline procedure (Methods, A. O. A. C., Seventh Edition).

Effect Upon Availability of Ascorbic Acid.—Traces of copper accentuate the oxidation of ascorbic acid, and copper when bound as a protein complex is still effective in accentuating this oxidation. This suggested that a high level of copper in the diet as potassium sodium copper chlorophyllin or of other copper salts might result in a vitamin C deficiency. An attempt was made to feed dry mix diets containing "chlorophyllin," copper sulfate, or copper gluconate, to guinea pigs, but the animals refused diets containing copper sulfate. By trial it was found that guinea pigs would not refuse these materials when supplied in water up to a copper content equivalent of 0.5% "chlorophyllin," (260 p. p. m. Cu). Subsequently 20 young female pigs weighing 150 to 200 Gm. each, were divided into four groups: one group was maintained as a control, the other groups received copper sulfate, copper gluconate, or "chlorophyllin" in all of their fluid intake (water). The animals were maintained for eleven weeks on a diet reasonably adequate in vitamin C, comprised of Rockland guinea-pig pellets and 70–75 Gm. of fresh cabbage per week. Food and water consumption did not markedly differ except in the first few weeks, during which the animals on the copper sulfate solution did not drink as freely. There was no clinical evidence of scurvy in any of the animals; no significant difference in survival; weight gain was better in the "chlorophyllin" animals, and not greatly different in the animals of the other groups. However, animals receiving copper sulfate and copper gluconate did not gain weight as rapidly during the initial period of test. At the termination of the eleventh week all animals were sacrificed and found to be without gross evidence of scurvy. Blood ascorbic acid was determined before sacrificing; there was no significant difference between the average figures of the several groups (Table XI).

TABLE XI.—ASCORBIC ACID CONTENT OF SERUM OR WHOLE BLOOD, mg. %

Diet	Rat Blood Serum Males	Rat Blood Serum Females	Guinea-Pig Blood, Females
"Chlorophyllin, 3%" (1,600 p. p. m. Cu)	0.43	0.40	0.96
Copper sulfate (1,600 p. p. m. Cu)	0.48	0.39	0.95
Copper gluconate (1,600 p. p. m. Cu)	0.12 0.55	0.57	1.05
Control (2 p. p. m. Cu)	0.47	0.39	0.90

Average of 5 rats, except copper gluconate where individual figures for 2 male rats is given. Average of 4 animals for guinea pigs.

Though rats do not require ascorbic acid in their diet, animals after being on the "chlorophylllin" diets for one year, and the copper sulfate and gluconate diets for forty weeks, were examined for serum ascorbic acid. No difference from the normal range

for rats was found, except that one of two animals which were receiving copper gluconate had a low serum ascorbic acid level.

PHOTOSENSITIZATION

To establish whether potassium sodium copper chlorophyllin would cause photosensitization if it were present as such in the blood or was degraded into sensitizing porphyrins, several routes of administration were followed and several means of excitement; that is, prolonged high intensity light (photoflood), short high intensity light (photoflash), and prolonged ultraviolet exposure (quartz mercury arc). Guinea pigs, mice, and rats were subjected to exposure by these means after having received the "chlorophyllin" by direct application to the skin, oral intake, intraperitoneal, or intravenous injection. Only albino animals were employed and these were in some instances shaved and washed before exposure. Neither general shock, pustulation, edema, or erythema developed except that erythema was produced by the quartz mercury arc in shaved animals upon prolonged exposure, including the controls. The guinea pig, which is quite susceptible to sensitization by many substances, was not abnormal in response even after repeated application of the material. Furthermore, animals which had received as much as 3% of "chlorophyllin" in their diet for over a year, responded no differently than did control animals. These experimental procedures failed to produce photosensitization.

DISCUSSION

Many investigators have shown that the administration of copper in any considerable dosage to the albino rat is followed by metal toxicity, an increased storage of copper, more especially in the liver, kidney and spleen, damage to these organs, and a high mortality. We have confirmed these findings and observed that copper, when organically combined as the gluconate, either more readily passes across the gastrointestinal membrane or is protected against inactivation by proteins, lipids, etc., present in the food. Thus it is stored more rapidly and to a greater extent, resulting in an increased mortality. Copper or other metallic salts may in this form offer a therapeutic advantage.

Equivalent daily amounts of copper administered as the complex potassium sodium copper chlorophyllin do not cause metal toxicity even when given over a more prolonged period. There is no storage of copper in the liver, kidney, or spleens of such animals comparable to that resulting subsequent to the administration of copper in the form of sulfate or gluconate. Therefore, though excessive amounts of copper cause liver damage when given in the form of a salt such as the sulfate or gluconate, or as powdered metallic copper, the same amount of copper complexed with "chlorophyll" is innocuous. This copper is not available for deposit in the tissues, though the chlorophyllin complex is transported in the plasma, when a sufficient concentration of the material is fed in the diet. However, the full complement of copper for the amount of "chlorophyllin" in the plasma is not found. Only about 1/6 of the theoretical copper is present.

When there is a high intake of available copper as sulfate, etc., as distinguished from the firmly bound copper of "chlorophyllin" there is an over-all high level of storage in the tissues. However, there also appears a great many individual peculiarities. An occasional animal may store a tremendous quantity in some one tissue, such as the liver, yet another animal on the same intake may store no more copper than a control animal on a blank diet. For instance, copper storage in the livers of the group on copper sulfate (530 p. p. m. Cu) range from 1–79 mg. copper per 100 Gm. of tissue. Neither an excessively high storage, nor an inordinately low storage of copper follows in other tissues of the same animals. There is therefore some individual peculiarity of a particular tissue that plays an unexplained role.

Corwin (8) in commenting upon photosensitization suggested that effective amounts of chlorophyll may not enter the blood stream and Zirm and Kilches (9) found no evidence of water-soluble chlorophyll derivatives in the blood or tissues when C^{14} labeled sodium magnesium chlorophyllin was administered orally. We have shown by spectrophotometric examination that at least a portion of the chlorophyll complex does enter the blood when a sufficient concentration is fed in the diet. However, photosensitization, which is often attributed to porphyrins did not follow and it was not possible to develop light sensitive animals by other means of administration of the material.

Blum (25) also observed that the presence of porphyrins does not necessarily indicate sensitivity to light and that sensitivity to light is not always accompanied by the presence of porphyrins.

Sensitizing porphyrins are absent though porphyrins are extracted from the 5 and 10% HCl fecal fractions as evidenced by fluorescence in the ultraviolet region following the procedure of Brugsch and Sheard (26). But phylloerythrin appears to be absent, as absorption peaks do not occur at 520 mμ (Fig. 5). Furthermore, the porphyrin content of the feces from rats upon the control diet is not materially higher than that of the rats receiving 3% of the "chlorophyllin."

Potassium sodium copper chlorophyllin which is water-soluble in its original form is excreted in the feces in a water-insoluble and essentially organic solvent insoluble form. It has apparently exchanged its potassium and sodium and substituted calcium. Alkali does not reconvert it and the copper apparently remains complexed within the structure. This may in part explain the excellent survival over a period of nearly two years, of animals receiving "chlorophyllin" not withstanding the inordinately high amount of the copper complex which was purposely fed. Survival was even better than the survival of those animals on the blank or control diet, and may be compared with the 90% mortality of male rats upon copper gluconate, and a high mortality among the high level copper sulfate animals, all of which substantiate the essential nontoxicity of the potassium sodium copper chlorophyllin.

We presume it is by reason of the firmness of this copper complex that a high intake of copper in this form is innocuous. Further, the complexed copper not being readily removed from within the structure, the conversion of the potassium sodium copper

chlorophyllin as ingested to other chlorophyll fractions, is blocked. As the magnesium in chlorophyll or in a true chlorophyllin is readily removed, this physical-chemical blocking may not exist for these compounds.

Potassium sodium copper chlorophyllin is, under these circumstances, apparently essentially excreted as an insoluble calcium copper chlorophyllin.

SUMMARY

The inclusion of up to 3 per cent of potassium sodium copper chlorophyllin in the diet of albino rats over their life span resulted in no indication of toxicity, in that:

1. The growth rate, survival, blood and urine factors, and ability to conceive, were normal.

2. There was no gross or microscopic pathology attributable to the "chlorophyllin."

3. There was no evidence of metal toxicity, and the livers, kidneys, and spleens, did not store increased amounts of copper, compared with that stored by similar tissues of animals receiving equilivalent quantities of copper sulfate or copper gluconate.

4. Photosensitization did not occur, nor was it possible to incite sensitization by intraperitoneal or local application of the "chlorophyllin."

During the study it was also observed that:

5. Plasma contains "chlorophyllin" when a sufficient amount is fed in the diet.

6. Plasma contains additional copper under the same circumstances, but only to the extent of about 1/6 of that theoretically expected.

7. Copper gluconate is more readily absorbed and deposited in the tissues when administered orally than is an equivalent amount of copper as the sulfate.

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WHO MAKES IT?

The Registry of Rare Chemicals, Armour Research Foundation of the Illinois Institute of Technology, 35 West 33rd Street, Chicago 16, Ill., seeks information on sources of supply of the following chemicals:

Aluminum nitride
Silver hyponitrite
Sodium telluride
Phosphonium iodide
Diethylgermanium iodide
1,4-Cyclohexanediamine
Anthracene-9,10-dicarboxylic acid
2-Chlorocyclopentene-1
7-Nitrohydrindene
2,3,5,6-Tetramethylbenzoic acid
2,5-Dihydroxyphenylalanine
2,4,2′,4′-Tetrahydroxydiphenyl
2,3-Dichlorobenzyl alcohol
Epifluorohydrin
10-Methyl-1,2-benzanthracene
β-Chlorolactic acid
Murexine
Tangeretin
Nobiletin
Muscarine

Composition of Gum Turpentines of Pines

XXI. A Report on Pinus quadrifolia from Southern California, Pinus lumholtzii from Durango, Mexico, and Pinus caribaea from Nicaragua*

By P. M. ILOFF, Jr., and N. T. MIROV†

Pinus quadrifolia **Parl. is called Parry pinyon. According to some botanists (6) it is one of the three varieties of *P. cembroides*. It grows in dry, almost desert, localities of Southern California and of the northern part of Lower California, Mexico. When it comes in contact with the other pinyon pines, apparently it crosses with these, as intermediate forms between *P. cembroides* and other pinyons are quite common.**

The oleoresin sample of this pine was obtained in August, 1952, with the assistance of officers of the San Bernardino National Forest, about four miles east of Anza, Riverside County, California, at an elevation of about 4,000 feet.

YIELD OF OLEORESIN was rather large for the dry environment—about four ounces per cup per tapping. The oleoresin samples (from eleven trees) were treated separately; they were heated gradually to 190° under gradually diminishing pressure; at the end of the operation when all turpentine had been distilled, the pressure was 0.5 mm. Average yield of turpentine was 23.5 per cent. Physical characteristics of the turpentine samples varied within these limits:

density, d_4^{23} = from 0.8609 to 0.8668
index of refraction, n_D^{20} = from 1.4732 to 1.4787
specific rotation $[\alpha]_D$ = from +6.2 to +32.7

The composed turpentine, i. e., a mixture of all eleven individual samples, had the following physical characteristics: $d_4^{23.5}$, 0.8629; n_D^{24}, 1.4742; $[\alpha]_D^{23}$, +24.8. A charge of 538 Gm. of the turpentine was distilled through a 90-cm. Vigreux column equipped with a heated jacket. A reflux ratio of 5:1 was used. The results of the distillation are shown in Table I. Fraction A was charged to a 90-cm. long, 12-mm. inside diameter Todd column packed with glass helices. After 57.8 per cent of the original 538 Gm. had been received, fraction B was added to the pot and the distillation was continued at reduced pressure. A 10:1 reflux ratio was maintained. Fraction C was distilled through a 90-cm. long, 5-mm. inside diameter Todd column packed with a wire spiral. A reflux ratio of 10:1 was maintained. The results of the distillation are shown in Table II.

The physical properties of fraction 1 are close to the properties for pure α-pinene.

Crude *dl*-α-pinene nitrosochloride (0.4 Gm.) was prepared from 7 ml. of fraction 1. After two recrystallizations from chloroform by addition of cold methanol it melted at 103–104°. No depression in melting point occurred upon admixture with authentic *dl*-α-pinene nitrosochloride.

dl-α-Pinene nitrosochloride was also prepared from fraction 2.

Fraction 6 had physical properties similar to those of ocimene. One milliliter of this fraction left to stand open in the air for two days turned gummy. Ocimene is known to behave in a similar manner (8).

	B. P.	n_D	d_4^{25}	$[\alpha]_D^{25}$
Ocimene (9)	63° at 10 mm.	1.4836^{25}	0.7947	0
Fraction (6)	62–64° at 13 mm.	1.4832^{23}	0.7985	+5.5

Three grams of the oil from fraction 6 was reduced with sodium in alcohol. The reduction mixture was treated with water and extracted with ether. The ether extract was dried and bromine was added dropwise, keeping the solution in an ice-salt bath. A bromide was formed which, after several recrystallizations from ethyl acetate in the refrigerator, melted at 93–94°. An analysis showed: C, 26.36%; H, 3.90%;

TABLE I.—PRELIMINARY DISTILLATION OF GUM TURPENTINE OF PINUS QUADRIFOLIA

Fraction	Boiling Point	Pressure, mm.	% of Distillate
A	50–57	16	64.8
B	57–90	16	5.6
C	90–142	16	18.5
D	135–36	10	1.6
E	113	1.0	1.0
Pot residue	...	...	6.9
Losses	...	...	1.2

* Received March 16, 1954, from the Institute of Forest Genetics, California Forest and Range Experiment Station, maintained by the Forest Service, U. S. Department of Agriculture, in cooperation with the University of California, Berkeley.

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TABLE II.—FRACTIONAL DISTILLATION OF TURPENTINES OF PINUS QUADRIFOLIA, PINUS LUMHOLTZII, AND PINUS CARIBAEA FROM NICARAGUA

Fractions	Pressure, mm.	Boiling Range, °C.	Distillate, %	Density	Index of Refraction	Specific Rotation
			Pinus quadrifolia (538 Gm. used)			
				d_4^{25}	n_D^{23}	$[\alpha]_D^{25}$
1	760	154–155.5	57.6	0.8533	1.4644	+48.2
2	13	40.5–47	3.7	0.8521	1.4653	+44.8
3	13	47–53	0.5	0.8326	1.4699	+18.2
4	13	53–59	1.2	0.8116	1.4712	+7.6
5	13	59–62	0.5	0.8109	1.4718	+11.8
6	13	62–64	1.8	0.7985	1.4832	+5.5
7	13	64–67	0.5	0.8140	1.4827	+9.9
				$d_4^{26.5}$	$n_D^{20.5}$	
8	12	82–85	0.2	0.8793	1.4660	−10.0
9	12	85–88	0.6	0.8803	1.4440	+0.4
10	12	88–100	0.2	0.9073	1.4517	+11.0
11	12	100–106	0.3	0.9151	1.4630	+3.9
12	12	106–110	0.4	0.9065	1.4752	−5.1
13	12	110–115	1.9	0.8984	1.4812	−2.3
14	12	116–116	4.7	0.9050	1.4925	+5.1
15	12	116–121	1.6	0.9053	1.4972	+8.3
16	12	121–130	1.7	0.9005	1.5030	+8.0
17	12	130–131	1.1	0.9096	1.5083	+3.5
18	12	131–132	2.8	0.8990	1.5075	+26.5
19	12	Above 132	0.3	0.8990	1.5082	+27.8
D	...	...	1.6	0.9021	1.5079	+24.2
E	...	...	1.0	...	1.5108	...
Pot residue and losses	...	...	15.8	...	...	...
			Pinus lumholtzii (646 Gm. used)			
				d_4^{23}	n_D^{20}	$[\alpha]_D^{24}$
1	760	146–154.5	0.7	0.8348	1.4633	+24.6
2	760	154.5–155	11.4	0.8543	1.4932	+24.6
3	760	155–157.5	2.5	0.8517	1.4935	+20.5
4–18	760	157.5–174	7.7	0.8483–0.8370	1.4571–1.4728	+14.6 to −88.8
19	760	174–175	0.9	0.8382	1.4730	−99.1
20	760	175–176	3.1	0.8387	1.4731	−110.2
21	760	175–176.4	7.4	0.8386	1.4731	−116.9
22	14	63–64	16.9	0.8391	1.4731	−120.9
23	14	64–64	11.3	0.8388	1.4733	−124.0
24	14	64–65	27.3	0.8399	1.4733	−122.8
25	14	65–66	0.5	0.8397	1.4751	−119.2
26–31	14	66–94	2.4	0.8455–0.9037	1.4765–1.4998	
32	14	94–97	0.8	0.9460	1.5130	−7.1
33	14	97–98	1.2	0.9589	1.5182	−1.2
34	10	97–100	0.4	0.9491	1.5061	+12.7
35	4	93–100	0.7	0.9390	1.5030	+38.2
Pot residue	...	...	2.1	...	...	...
Loss	...	...	2.7	...	...	...
			Pinus caribaea from Nicaragua (51.6 Gm. used)			
				d_4^{25}	n_D^{25}	$[\alpha]_D^{25}$
1	11	40–40	38.9	0.8546	1.4625	−34.6
2	11	40–41	14.7	0.8545	1.4637	−35.1
3	11	41–44	2.1	0.8554	1.4653	−31.6
4	11	44–50	2.9	0.8544	1.4681	−23.4
5	11	50–52	2.0	0.8565	1.4700	−8.6
6	11	52–55	4.9	0.8556	1.4720	+2.8
7	11	55–57	3.1	0.8533	1.4737	+1.2
8	11	57–59	2.7	0.8466	1.4768	−4.7
9	11	59–61	2.1	0.8419	1.4792	−7.4
10	11	61–90	2.0	0.8871	1.4853	−9.2
11	11	90–96	3.3	0.9329	1.4906	−11.6
12	11	96–108	2.7	0.9370	1.4880	−14.2
13	11	108–120	7.0	0.9350	1.4987	+37.4
Pot residue and loss	...	...	11.6	...	...	...

calc. for $C_{10}H_{18}Br_4$: C, 26.22%; H, 3.96%. The literature value for the melting point of dihydroöcimene tetrabromide is 87–88° (2). The ultraviolet absorption spectrum showed a maximum at λ max. 235 mμ in absolute alcohol. The literature value for ocimene is λ max. 237 mμ in iso-octane (10). Probably the high proportion of pot residue received at the end of the distillation was due to polymerization of ocimene.

Fraction 14 had the molecular refractivity R_D 65.4, indicating a dicyclic sesquiterpene with two double bonds. There is a medium maximum in the infrared at 1,646 cm.$^{-1}$ and a strong maximum at 888 cm.$^{-1}$ which indicates the presence of a $R_1R_2C{=}CH_2$ group.

A dehydrogenation with sulfur was carried out on 4 Gm. of oil from fraction 14 but no product was received.

A hydrochloride was prepared from 3.0 ml. of fraction 14, and 0.44 Gm. of crude crystals was obtained. After several recrystallizations from glacial acetic acid, the melting point was 117–118° and showed no depression with cadinene hydrochloride. The optical rotation of the hydrochloride was $[\alpha]_D^{23.5}$, −39.0 (c = 5.0 in $CHCl_3$).

Fraction 17 had the molecular refractivity R_D = 66.6, indicating a dicyclic sesquiterpene with two double bonds.

A hydrochloride was prepared from 3 Gm. of fraction 17. About 1.1 Gm. of crude needle-like crystals was received. After several recrystallizations from glacial acetic acid the hydrochloride melted at 117–118° and had the optical rotation: $[\alpha]_D^{23}$, −43.1 (c = 9.4 in $CHCl_3$).

Fraction 18 had the molecular refractivity R_D = 67.4 which is near that of a monocyclic sesquiterpene.

Turpentine was obtained from a batch of 330 Gm. of oleoresin. At the end of distillation the temperature was 180° and the pressure was 0.5 mm. of mercury.

The gum turpentine had the following properties: d_4^{25}, 0.8670; n_D^{24}, 1.4708; $[\alpha]_D^{23}$, −20.6. A batch of 51.6 Gm. of the turpentine was distilled in a 90-cm. long, and 5-mm. inside diameter wire spiral column at a pressure of 11 mm. A reflux ratio of 10:1 was maintained until the temperature reached 61° after which a reflux ratio of 2:1 was used. Results of the fractionation are shown in Table II.

The physical properties of the first three fractions were close to those of α-pinene. One and six-tenths grams of α-pinene nitrosochloride was prepared from 7 ml. of fraction 1. After three recrystallizations *dl*-α-pinene nitrosochloride with a constant melting point of 103–104° was obtained. When it was mixed with authentic *dl*-α-pinene nitrosochloride, the melting point was not depressed. The first four fractions were primarily *l*- and *dl*-α-pinene.

Fractions 6 and 7 had the sweet odor of Δ-^{3}-carene. The physical properties and rotation also indicate the presence of *d*-Δ^3-carene. A nitrosate was prepared from fraction 7 and, after two recrystallizations from chloroform by addition of cold methanol, melted at 143–144° and showed no depression in melting point on admixture with Δ^3-carene nitrosate derived from *Pinus ponderosa* turpentine.

The increase in refractive index and decrease in density for fractions 8 and 9 suggest the presence of *l*-β-phellandrene.

A nitrosite was prepared from fraction 8 which, after three recrystallizations from chloroform by addition of cold methanol, melted at 97–98°.

A nitrosochloride was prepared from fraction 9 using the method of West (11). The nitrosochloride melted at 99–100° and, after recrystallization from chloroform by addition of cold methanol, melted at 105–106°. *dl*-α-Pinene nitrosochloride with a similar melting point produced a 5–6° depression in melting point in a mixed melting point. A mixed melting-point determination using the nitrosochloride from fraction 9 and a nitrosochloride with the same melting point, prepared from *l*-β-phellandrene from *Pinus contorta* turpentine showed no depression.

The properties of fraction 13 suggested that the fraction was mainly *d*-longifolene. Seven-tenths gram of crude sesquiterpene hydrochloride was prepared from 1.4 Gm. of fraction 13. After two recrystallizations from glacial acetic acid, crystals were received which melted at 59–59.5° and showed no depression of melting point on admixture with longifolene hydrochloride derived from *Pinus apacheca* turpentine.

To sum up, the turpentine of *Pinus caribaea* from Nicaragua consisted of 59–60% *l*- and *dl*-α-pinene; 10% *d*-Δ^3-carene; 5% *l*-β-phellandrene; 13–14% tails of which the major constituent is *d*-longifolene; and about 11% polymerized pot residue and losses. An analysis of a larger sample of *Pinus caribaea* turpentine would be desirable.

Four grams of oil from fraction 18 was mixed with 1.88 Gm. of sulfur and dehydrogenated according to the method of Ruzicka and Meyer (5). The oil received was distilled at reduced pressure. The distillate was treated with 1 Gm. of trinitrobenzene dissolved in hot alcohol. The mixture was warmed on the steam bath until the reactants were dissolved, and on cooling 1.6 Gm. of yellow crystals was received. After two recrystallizations from hot alcohol the crystals melted at 111–112° and further recrystallization did not raise the melting point. Admixture with authentic cadalene trinitrobenzene adduct caused no depression in melting point.

Fraction 18 showed strong absorption maxima in the infrared region at 1,651 cm.$^{-1}$ and at 888 cm.$^{-1}$. This is interpreted as indicating the presence of an $R_1R_2C{=}CH_2$ group. The average value for C=C stretching for $R_1R_2C{=}CH_2$ is 1,653 and for C—H out-of-plane wagging is 890 (7). Judging from the above evidence, fraction 18 is a cadalene type sesquiterpene possessing an $R_1R_2C{=}CH_2$ group.

To sum up, the turpentine of *Pinus quadrifolia* contains 62 per cent *d*- and dl-α-pinene, 3.8 per

cent ocimene, 1.7 per cent intermediate unidentified mixture; 16.7 per cent sesquiterpenes, the main components of which are two dicyclic sesquiterpenes that yield cadinene hydrochloride on treatment with dry hydrochloric acid and a cadalene type sesquiterpene (possibly monocyclic), and 15.8 per cent pot residue and losses.

Pinus lumholtzii Rob. et Fern. is a picturesque Mexican pine, with bright green drooping foliage (6). It is called *pino triste* (sad pine) or *pino barba caida* (drooping beard pine). It grows in the Western Mexican states from Chihuahua to Jalisco. A sample of oleoresin of this pine was obtained through the courtesy of forest engineer Cenobio E. Blanco. The sample was collected at an elevation of about 8,500 feet near El Salto, Durango, and was accompanied with foliage and branches, now kept for further reference purposes. In order to obtain the turpentine, a batch of about 4,000 Gm. of oleoresin was heated gradually, under a reduced pressure, so that at the end of distillation the temperature reached 190°, and the pressure was reduced to 1 mm. of mercury. Yield of turpentine amounted to 18%.

The turpentine possessed the following characteristics: d_4^{23}, 0.8455; n_D^{23}, 1.4723; $[\alpha]_D^{24}$, −81.3.

The physical constants of fraction 2 indicated that it consisted of *d*- and *dl*-α-pinene. A nitrosochloride was prepared, which was recrystallized to a constant melting point of 103–104° by addition of cold methanol to a chloroform solution of the nitrosochloride. The crystals showed no melting-point depression when mixed with an authentic sample of *dl*-α-pinene nitrosochloride.

Fraction 3 had the properties of *d*- and *dl*-α-pinene mixed with a small amount of *l*-limonene. Fractions 4 to 18 had properties indicating mixtures of *d*- and *dl*-α-pinene and *l*-limonene. When the refractive indices and densities of these fractions were plotted against each other on Cartesian coordinates, the points fell close to a straight line between the properties of α-pinene and those of limonene. This indicates that the fractions are mixtures of α-pinene and limonene.

By addition of bromine, limonene tetrabromide, m. p. 103°, was prepared from fraction 18.

A tetrabromide, m. p. 102–103°, was prepared from fraction 23. A mixture with a sample of limonene tetrabromide, m. p. 103–104°, melted at 103°.

The fractions 26 to 31 were transition fractions. The densities and refractive indices were plotted against each other on Cartesian coordinates and the point for each fraction was very close to a straight line drawn between the point for limonene and the point for fraction 33. From the physical constants and distillation behavior it was indicated that a pure compound was separated in fraction 33. The properties and odor indicated methyl chavicol:

Methyl chavicol (*a*): d_{25}^{25}, 0.9600; n_D^{22}, 1.5137; $[\alpha]_D^{25}$, 0.0.

Fraction 33: d_4^{23}, 0.9589; n_D^{20}, 1.5182; $[\alpha]_D^{23}$, −1.2.

[(*a*) From Hasselstrom, T., and Hampton, B., *J. Am. Chem. Soc.*, *60*; 3068(1938).]

Five grams of oil from fraction 33 was added to a solution of 3.3 Gm. of potassium permanganate in 330 ml. of water and 3.5 ml. of glacial acetic acid and shaken in an ice bath for three hours. The mixture was then made alkaline with potassium carbonate and filtered. The clear solution was reduced to 1/3 volume by boiling, allowed to cool, and acidified with excess of dilute sulfuric acid. The acid solution was extracted with three separate 25-ml. portions of ether. By evaporation of the ether 0.4 Gm. of crude crystals was received. These crystals were pressed on a clay plate and, after several alternate recrystallizations from hot water and from hot hexane, white plates with a constant melting point of 80–82° were received. An analysis showed: C, 65.29; H, 6.16%; calcd. for $C_9H_{10}O_4$; C, 65.05; H, 6.07%.

The literature value for the melting point of homoanisic acid is 84–85° (1). Fifty milligrams of the homoanisic acid was added to 10 ml. of water to which 1 ml. of glacial acetic acid and 77 mg. of chromic anhydride had been added. The mixture was heated on the steam bath for four hours; upon cooling, crystals were formed. The crystals were recrystallized from water several times to yield white needles, m. p. 184.5–185.5°, which showed no depression in melting point upon admixture with authentic anisic acid.

Fraction 35 had the physical properties of a tricyclic sesquiterpene with the density being somewhat high due to the presence of some methyl chavicol. From 2 Gm. of oil, 0.7 Gm. of a hydrochloride was prepared. After three recrystallizations from glacial acetic acid the crystals melted at 59° and were not depressed in melting point by admixture of an authentic hydrochloride of *d*-longifolene.

To sum up, *Pinus lumholtzii* gum turpentine contained: 16% *d*- and *dl*-α-pinene, 75% *l*-limonene, 2–3% methyl chavicol, 1% *d*-longifolene, 2% unidentified sesquiterpenes, and 4.8% pot residue and loss.

Pinus caribaea Morelet grows in Bahama Islands, Western Cuba, Isle of Pines, Honduras, British Honduras, Guatemala, and Nicaragua. Until recently our Southeastern slash pine, whose official name now is *Pinus elliottii* (3), was also known under this name. A small sample of oleoresin of *Pinus caribaea* was obtained through the courtesy of Professor James J. Parsons of the University of California. The sample was collected in April, 1953, at Karwala, near the mouth of the Rio Grande, about 50 miles north of Bluefields, Nicaragua.

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Composition of Gum Turpentines of Pines

XXII. A Report on Pinus rudis and P. hartwegii from Mexico and P. insularis from Philippines*

By P. M. ILOFF, Jr., and N. T. MIROV

P. rudis **grows over a large territory in Mexico. On the basis of the composition of its turpentine which differs from that of *P. montezumae*, *Pinus rudis* should be considered a separate species. *Pinus hartwegii*, another Mexican pine, grows at elevations ranging from 8,400 to 11,500 feet. Its chemical composition differs so from that of *P. montezumae* that these investigators are inclined to consider it also as an independent species. Oleoresin of *Pinus insularis* was used experimentally to determine the turpentine content which was found to be higher than previously reported from the Philippines and Burma where *Pinus insularis* has long been exploited for turpentine and resin. The discovery of *l*-β-phellandrene was unexpected as it has never been reported in Asiatic pines.**

PINUS RUDIS

Pinus rudis Endl., in the classification of Shaw (7, p. 64), is called *P. montezumae* var. *rudis*. The Mexican botanist Martinez (4, p. 256), however, considers it as an independent species. We have observed this pine in Mexico and are inclined to accept its specific rank. *P. rudis* and *P. montezumae* are related and apparently they hybridize naturally; both species and many intermediate forms are often found in the same locality. *P. rudis* grows over a large territory from Coahuila and Tamaulipas to Michoacan and farther east to Puebla and Oaxaca.

The oleoresin sample of this pine was collected by Jesse P. Perry, Jr., Rockefeller Foundation, Mexico City, along the new Texcoco-Veracruz Highway, on the border between the states of Mexico and Tlaxcala.

EXPERIMENTAL

A batch of 2,439 Gm. of oleoresin was distilled under reduced pressure so that at the end of distillation, temperature was 183° and pressure was 0.5 mm. of mercury. The 554 Gm. of turpentine obtained in this manner amounts to 22.7% of the weight of the oleoresin.

The turpentine of *P. rudis* had the following physical characteristics: d_4^{24}, 0.8627; n_D^{23}, 1.4734; $[\alpha]_D^{24}$, −11.1°.

A batch of 518 Gm. of the turpentine was distilled in a 90-cm., 25-mm. inside diameter, Todd column packed with glass helices. A reflux ratio of 10:1 was maintained. Results of the distillation are shown in Table I.

Fraction 2 was tested for the presence of α-pinene. A nitrosochloride was prepared in good yield; after three recrystallizations the derivative melted at 103–104° and showed no depression in melting point on admixture with authentic *dl*-α-pinene nitrosochloride.

A nitrolpiperidide was prepared from the nitrosochloride and, after two recrystallizations from 90% alcohol, melted at 120–120.5°.

The properties of the subsequent fractions indicate mixtures of α- and β-pinene. Fractions 9, 10, and 11 have properties very close to those of pure β-pinene (9).

Fraction 10 was tested for β-pinene by oxidation with potassium permanganate. The nopinic acid received was recrystallized from benzene until it melted at 125–125.5° and showed no depression in melting point on admixture with authentic nopinic acid.

A tetrabromide was made from fraction 14. After three recrystallizations from ethyl acetate it melted at 124°. For comparison, dipentene tetrabromide melting at 124° was prepared from a mixture of known *d*-limonene and *l*-limonene. A mixture of the two tetrabromides melted at 124°.

Fraction 17 treated with dry HCl gas in ether solution yielded no hydrochloride.

Judging from the distillation behavior and physical properties of the fractions, the gum turpentine of *Pinus rudis* consisted of 32% *d*- and *dl*-α-pinene, 62% *l*-β-pinene, 1% dipentene, and about 1% higher boiling substances. On the basis of the composition of its turpentine, which differs from that of *P. montezumae* (5), *Pinus rudis* should be considered as a separate species.

PINUS HARTWEGII

Pinus hartwegii Lindl. is another Mexican pine which is considered by Shaw as a mere variety of *P. montezumae*. Standley (8), Robertson (6) and Martinez (4), however, consider it as an independent species. It grows in the State of Mexico and adjacent States and also it has been reported from Veracruz, Oaxaca, and Chiapas. It occurs in higher elevations than any other pine of the world, being found in its upper limit near the line of permanent snow, higher than 13,100 feet (4,000 meters). On the slopes of the two famous Mexican volcanoes, Popocatepetl and

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Iztaccihuatl, *Pinus hartwegii* forms pure stands at elevations ranging from 8,400 feet to 11,500 feet.

EXPERIMENTAL

Turpentine of *P. hartwegii*, obtained by steam distillation of oleoresin, was previously analyzed by Iriarte (3). He obtained 27.7% of turpentine which possessed the following characteristics: d_4^{24}, 0.8637; n_D^{22}, 1.4653; $[\alpha]_D^{20}$, +2.5°. The turpentine was fractionated by means of a 15-cm. column and was found to contain at least 65% of *d*, *dl*-α-pinene and a small amount of *l*-limonene.

For the present experiments, a sample of oleoresin was collected by Jesse P. Perry, Jr., in a pure stand of *P. hartwegii* near the timberline on the slopes of Popocatepetl.

A batch of oleoresin weighing 1,144 Gm. was heated *in vacuo* so that at the end of distillation the temperature reached 180° and pressure was reduced to 0.5 mm. of mercury. By this means 340 Gm. of turpentine, 29.7% of the weight of oleoresin, was obtained. The turpentine possessed the following characteristics: d_4^{23}, 0.8480; n_D^{23}, 1.4705; $[\alpha]_D^{23}$, −48.7°.

A batch of 334 Gm. of the turpentine was distilled in a 90 cm., 12-mm. inside-diameter, Todd column packed with glass helices. A reflux ratio of 10:1 was maintained. The results of the distillation are shown in Table I.

Fraction 1 has physical properties close to those of α-pinene. From 7 ml. of fraction 1, 0.85 Gm. of crude *dl*-α-pinene nitrosochloride was prepared. After recrystallization, it melted at 103–104° and showed no depression in melting point on admixture with authentic *dl*-α-pinene nitrosochloride. A nitrolpiperidide, m. p. 119–120°, was prepared from the above nitrosochloride. The nitrolpiperidide showed no depression in melting point on admixture with authentic *dl*-α-pinene nitrolpiperidide.

Fraction 6 was tested for β-myrcene by warming part of it with maleic anhydride, and a small amount of adduct was isolated by fractional crystallization from petroleum ether at 0°. The adduct melted at 33–34° and showed no depression in melting point on admixture with the maleic anhydride adduct of β-myrcene. Although the characteristic ultraviolet absorption maximum was masked by some other substance, it was concluded that this fraction contained a small amount of β-myrcene. Alkaline permanganate oxidation of fraction 7 yielded only *cis*-caronic acid, possessing m. p. 175–176° Evidently it came from Δ^3-carene. Fractions 8 and 9 had properties close to those of Δ^3-carene. A nitrosate was prepared from fraction 8. The nitrosate melted at 145–145.5° and showed no depression in melting point on admixture with authentic Δ^3-carene nitrosate.

Fractions 10 and 11 were transition fractions, containing considerable amounts of *l*-limonene. Fraction 13 had the highest rotation of the limonene fractions. A tetrabromide melting at 103–104° was prepared from fraction 13. The tetrabromide showed no depression in melting point on admixture with authentic *l*-limonene tetrabromide.

Fractions 16 to 18 were apparently of a transition character, possibly having some terpenes in such minute quantities as to make their identification impossible.

The high density of fraction 19, along with the odor, refractive index, and low rotation, indicated the possible presence of methyl chavicol. Oxidation by permanganate (1) at 0° and extraction of the acid fraction with hexane yielded crystals which, after several recrystallizations alternately from water and hexane, melted at 83–84° and showed no melting point depression on admixture with homoanisic acid.

By treatment with dry HCl gas in ether solution, a hydrochloride was prepared from fraction 21. After recrystallization from glacial acetic acid, the hydrochloride melted at 59–60° and showed no depression in melting point on admixture with authentic longifolene hydrochloride.

To sum up, the gum turpentine of *P. hartwegii* consisted of 28% *d*- and *dl*-α-pinene; 1% β-myrcene; 5% *d*-Δ^3carene; 56% *l*-limonene; 3% methyl chavicol; and 1–2% *d*-longifolene. The difference between Iriarte's results and ours were probably due to the difference in methods used. We employed a more efficient fractionating column. Chemical composition of turpentine of *P. hartwegii* is so different from that of *P. montezumae* (5), that we are inclined to agree with Standley, Martinez, and Robertson, in considering *P. hartwegii* as an independent species.

PINUS INSULARIS

Pinus insularis Endl. is a pine of the Philippines and of northern Burma. It has been exploited for turpentine and rosin for a long time. In 1931, DeSantos, *et al.* (2), reported that the yield of (commercial?) turpentine (presumably steam-distilled) varied from 15.33 to 17.23 per cent. Analysis of the turpentine was limited only to the identification of α-pinene by preparing pinene hydrochloride. Apparently this turpene amounted to 75 or 80 per cent of the total turpentine.

EXPERIMENTAL

In our experiments, we used oleoresin received together with herbarium specimens in 1952 from Dr. Florencio Tamesis, Director of the Philippine Forestry Bureau. A batch of 2,000 Gm. of oleoresin was distilled under reduced pressure. At the end of the operation, temperature of the oleoresin was 190° and pressure was 3 mm. of mercury. The rosin remaining in the pot was very hard and brittle. The amount of turpentine in the oleoresin was 20.0%.

The turpentine had the following characteristics: d_4^{24}, 0.8586; n_D^{22}, 1.4685; $[\alpha]_D^{24}$, +31.2°.

A batch of 381 Gm. of the turpentine was distilled in a 90-cm., 12-mm. inside-diameter Todd column packed with glass helices. A reflux ratio of 10:1 was maintained. Results of the distillation are shown in Table I.

α-Pinene.—The physical constants of the first three fractions were very close to those of pure

TABLE I. FRACTIONAL DISTILLATION OF TURPENTINES OF PINUS RUDIS, P. HARTWEGII, AND P. INSULARIS

Fractions	Pressure, mm.	Boiling range, °C.	Distillate, %	Density	Index of Refraction	Specific Rotation
		Pinus rudis (518 Gm. used)				
				d_4^{24}	n_D^{23}	$[\alpha]_4^{24}$
1	760	153–157	1.6	0.8528	1.4665	+2.1
2	760	157–158	17.6	0.8530	1.4662	+2.7
3	760	158–160	11.8	0.8544	1.4673	−0.2
4	760	160–161	3.1	0.8585	1.4704	−6.7
5	760	161–162	4.1	0.8601	1.4719	−10.2
6	760	162–163	4.5	0.8588	1.4731	−12.7
7	760	163–164	7.0	0.8613	1.4747	−15.3
8	760	164–165	7.3	0.8613	1.4756	−18.0
9	760	165–165	19.8	0.8648	1.4773	−22.2
10	760	165–165	13.7	0.8654	1.4777	−22.8
11	11	47–50	2.7	0.8656	1.4777	−20.8
12	11	50–55	1.0	0.8580	1.4766	−15.9
13	11	55–57	0.4	0.8481	1.4773	−10.9
14	11	57–64	0.6	0.8426	1.4781	−7.4
15	11	64–84	0.3	0.8530	1.4797	−3.5
16	11	84–94	0.6	0.9078	1.4791	−0.4
17	11	94–120	0.8	0.9572	1.4811	−17.6
Pot residue and loss			3.1			
		Pinus hartwegii (334 Gm. used)				
				d_4^{25}	n_D^{25}	$[\alpha]_D^{25}$
1	760	155–156	25.0	0.8549	1.4647	+40.4
2	760	156–158	1.8	0.8514	1.4648	+39.8
3	760	158–161	1.1	0.8509	1.4657	+32.6
4	760	161–165	0.8	0.8483	1.4669	+6.9
5	760	165–167	2.2	0.8485	1.4680	0.0
6	760	167–169	1.6	0.8488	1.4690	−9.9
7	760	169–171	1.6	0.8483	1.4698	−26.0
8	11	47–51	1.1	0.8542	1.4686	+15.0
9	11	51–55	1.3	0.8548	1.4701	+4.2
10	11	55–57	2.5	0.8513	1.4714	−40.5
11	11	57–57	5.4	0.8458	1.4715	−80.5
12	11	57–57.5	28.0	0.8425	1.4716	−110.5
13	11	57.5–57.5	12.2	0.8387	1.4728	−126.0
14	11	57.5–58	3.6	0.8389	1.4717	−125.7
15	11	58–60	2.1	0.8379	1.4717	−123.0
16	11	60–73	1.0	0.8451	1.4756	−80.5
17	11	73–86	1.5	0.8706	1.4747	−20.9
18	11	86–89	0.7	0.9073	1.4879	−6.4
19	11	89–105	1.7	0.9489	1.5010	−1.7
20	11	105–112	0.5	0.9470	1.4959	+14.8
21	11	112–117	1.3	0.9377	1.4990	+30.1
Pot residue and loss			3.0			
		Pinus insularis (381 Gm. used)				
				d_4^{24}	n_D^{25}	$[\alpha]_D^{25}$
1	760	105–154	2.4	0.8547	1.4631	+42.6
2	760	154–155	22.3	0.8552	1.4632	+43.8
3	760	155–156	45.5	0.8560	1.4630	+42.9
4	760	156–157	3.4	0.8573	1.4638	+40.3
5	11	42–46	0.8	0.8588	1.4679	+26.6
6	11	46–50	0.7	0.8599	1.4714	+7.0
7	11	50–52	0.8	0.8581	1.4727	−10.5
8	11	52–55	1.2	0.8533	1.4729	−21.1
9	11	55–57	2.7	0.8445	1.4773	−25.3
10	11	57–58	3.1	0.8389	1.4819	−13.5
11	11	58–64	0.6	0.8429	1.4818	−8.5
12	11	64–68	0.2	0.8538	1.4835	−3.3
13	11	68–73	0.6	0.8593	1.4838	−4.0
14	11	73–86	0.6	0.8871	1.4808	−3.4
15	11	86–91	1.5	0.9174	1.4805	−1.3
16	11	91–98	1.3	0.9318	1.4813	+7.3
17	11	98–103	1.4	0.9339	1.4800	+13.7
18	11	103–114	0.4	0.9320	1.4840	+11.0
19	11	114–116	0.6	0.9315	1.4888	+1.9
20	11	Above 116	1.2	0.9032	1.4870	−4.2
Pot residue			4.8			
loss			3.9			

α-pinene. Fraction 1 was washed with potassium carbonate solution to remove the volatile acids before the physical constants were taken. From 7 ml. of fraction 2, a nitrosochloride was prepared and twice recrystallized from chloroform by addition of cold methanol. A yield of 0.6 Gm. melting at 103–104° and not depressed in melting point by authentic *dl*-α-pinene nitrosochloride was received. A nitrolpiperidide, which melted at 119–120° after recrystallization from ethyl acetate, was prepared from the nitrosochloride. The nitrolpiperidide showed no depression in melting point on admixture with authentic *dl*-α-pinene nitrolpiperidide.

β-Pinene.—The refractive indexes (25°) of the fractions were plotted as abscissae and the densities (25°) were plotted as ordinates; the resulting graph indicated the probable presence of β-pinene as well as β-phellandrene. A mixture of fractions 6 and 7 (5.7 Gm.) was oxidized with alkaline permanganate to sodium nopinate (0.12 Gm.). Nopinic acid melting at 125° was prepared from the sodium nopinate and showed no depression in melting point on admixture with authentic nopinic acid.

Phellandrene.—The distillation data indicated an accumulation of a pure substance in fraction 10. The properties are compared with the literature value for pure β-phellandrene below:

	Fraction 10	*l*-β-Phellandrene[a]
n_D^{25}	1.4819	1.4851
d	0.8389_4^{22}	0.8375_4^{25}
D	−13.5	−2.86
B. P.	57–58° at 11 mm.	70° at 20 mm.

[a] Davenport, J. B., Sutherland, M. D., and West, T. F., *J. Appl. Chem.*, **1**, 527(1951).

A nitrosite was prepared from fraction 10. After four recrystallizations from chloroform by addition of cold methanol, the nitrosite melted at 97–98° and was not depressed in melting point by addition of a similar nitrosite from β-phellandrene from *Pinus contorta*.

A nitrosochloride was prepared from fraction 10. The nitrosochloride was twice recrystallized from chloroform by addition of cold methanol and it melted at 103°. A mixture with a similar nitrosochloride prepared from β-phellandrene from *P. contorta* turpentine showed no depression in melting point.

Fraction 17 was treated with dry HCl gas. A hydrochloride was formed which melted at 47–48° after recrystallization from hexane.

To sum up, the turpentine of *P. insularis* contained 74% *d*- and *dl*-α-pinene, 3% *l*-β-pinene, 7% *l*-β-phellandrene. 5% polymerized pot residue (probably polymerized phellandrene), and 7% tails, including a sesquiterpene whose hydrochloride has a melting point of 47–48°. Therefore, chemical composition of *P. insularis* turpentine was found to be somewhat more complicated than reported by DeSantos (2). It was not surprising to find a small amount of β-pinene because this terpene is often found with α-pinene, but the presence of *l*-β-phellandrene was rather unexpected. β-Phellandrene has never been reported in Asiatic pines; so far it has been detected in four New World species (*P. contorta*, *P. coulteri*, *P. caribaea*, and *P. resinosa*). In all these pines β-phellandrene is found also in its *laevo*- form.

SUMMARY

P. rudis turpentine was found to consist of: *d*, and *dl*-α-pinene, 32 per cent; *l*-β-pinene 62 per cent; dipentene, 1 per cent; high-boiling fractions, 1 per cent. *P. hartwegii* turpentine was composed of: *d*, *dl*-α-pinene, 28 per cent; β-myrcene, 1 per cent; Δ^3-carene, 5 per cent; *l*-limonene, 56 per cent; methyl chavicol, 3 per cent; *d*-longifolene, 1 to 2 per cent. *P. insularis* turpentine contained: *d*, *dl*-α-pinene, 74 per cent; *l*-β-pinene, 3 per cent; *l*-β-phellandrene, 7 per cent; polymerized residue, 5 per cent; tails, including unidentified sesquiterpenes, 7 per cent.

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The Determination of Codeine Phosphate in Combination with Acetylsalicylic Acid, Phenacetin, and Caffeine by Nonaqueous Titration*

By M. PERNAROWSKI, L. G. CHATTEN, and L. LEVI

The powdered tablet is treated with a solution of phenol and chloroform, the tablet excipients are removed by filtration and the filtrate, after dilution with acetonitrile, is titrated potentiometrically for its codeine phosphate content with perchloric acid in *p*-dioxane. A standard deviation of ±0.64 per cent was obtained for five consecutive determinations using synthetic mixtures containing approximately 32 mg. of codeine phosphate. The method, therefore, can be considered to be as accurate as conventional acid-base titrations. Seven of the nineteen commercial preparations analyzed by this method contained substances which interfered in the titration of the alkaloidal salt. These tablets were assayed by suspending the powdered sample in water, adding dilute alkali, and extracting the codeine with chloroform. The volume of the chloroform solution was decreased to about 10 ml. by heating on a water bath and, after dilution with acetonitrile, the codeine content was determined by potentiometric titration.

MANY METHODS for the quantitative determination of codeine and codeine salts have been reported in the literature. Parke, *et al.* (1), however, were the first to describe a procedure for the determination of codeine phosphate in APC and C preparations. Although the method gives a quantitative estimation of the four active ingredients and, therefore, can be considered a useful analytical procedure, it requires equipment not ordinarily found in the average laboratory and suffers from a number of undesirable physicochemical features.

McElheny, *et al.* (2), described a method combining extraction by solvent partition, columnar chromatography, and potentiometric titration for the quantitative determination of codeine in opium and they reported its applicability to APC and C preparations. Unfortunately, no relevant experimental data were given and hence a fair evaluation of this extension of their method cannot be made. It would appear, however, that the procedure is fairly involved and not readily applicable to semimicro quantities of material.

EXPERIMENTAL

Apparatus.—(*a*) Beckman *p*H meter, Model G, or similar potentiometric titrator; (*b*) shielded glass electrode, Beckman No. 1190-80; (*c*) sealed calomel electrode, sleeve type, Beckman No. 1170-71; (*d*) microburet, calibrated to 0.01 ml.

Reagents.—(*a*) Phenol, reagent grade; (*b*) chloroform, A. C. S. grade; (*c*) acetonitrile, Eastman Spectro grade, or the equivalent; (*d*) dioxane, A. C. S. grade; (*e*) perchloric acid, 0.05 *N* in dioxane, prepared by dissolving approximately 4.2 ml. of 70–72% perchloric acid A. C. S. grade in 1 L. of dioxane. The solution is standardized by titration against N. B. S. potassium acid phthalate.

Procedure.—*Method I.*—Weigh and powder 20 tablets. To an aliquot representing approximately 32 mg. codeine phosphate, add 5 Gm. phenol and 10 ml. chloroform, stir to dissolve, and filter through a pledget of cotton, using 5 ml. of a solution of 5 Gm. phenol and 10 ml. chloroform for quantitative operation. Add 50 ml. of acetonitrile to the filtrate and titrate the solution potentiometrically, recording the millivolt readings for every 0.02 ml. of titrant added in the vicinity of the end point. The end point of the titration is taken at the milliliter reading where the ratio dE/dV is a maximum.

Method II. Transfer an aliquot of the powdered tablets equivalent to about 32 mg. codeine phosphate to a separatory funnel, add 25 ml. of distilled water, make alkaline with 4 ml. of 20% aqueous sodium hydroxide solution, and extract the opium alkaloid with five 20-ml. portions of chloroform. Wash the combined chloroform extracts with 10 ml. of distilled water, filter through a cotton pledget, evaporate on a water bath to about 10 ml., add 50 ml. of acetonitrile and titrate the solution potentiometrically, recording the millivolt readings every 0.01 ml. in the vicinity of the equivalence point.

Results.—Since the water content of codeine phosphate is a variable quantity, the sample used in these determinations was analyzed by Karl Fischer titration and found to contain 1.201 moles of water. The molecular weight of the codeine phosphate used was therefore considered to be 417.5.

In Table I are given the results obtained when samples of pure codeine phosphate, dissolved in the phenol-chloroform-acetonitrile solvent system, were titrated directly with 0.05 *N* perchloric acid in dioxane. A typical titration curve illustrating such an analysis is shown in Fig. 1. The potential break

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TABLE I.—ANALYSIS OF CODEINE PHOSPHATE BY METHOD I

Mg. Taken,	Mg. Recovered,
69.30	69.58
68.00	67.92
58.35	58.81
67.90	68.75
67.48	67.92
Mean Recovery, %	100.59
Standard Deviation, %	±0.50

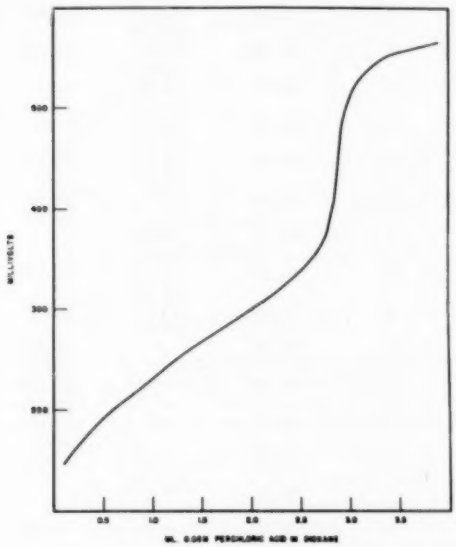

FIG. 1. TITRATION OF CODEINE PHOSPHATE IN PHENOL–CHLOROFORM–ACETONITRILE.

occurring at the end point is of considerable magnitude (dE/dV being approximately 800 to 1,000) and hence titrimetric equivalence can be detected with accuracy.

The method gave excellent results when applied to five commercial codeine phosphate tablets. The error between duplicates was of the order of 0.7% and in all of the assays sharp potential breaks were obtained at the end point.

Recoveries of codeine phosphate from synthetic APC and C mixtures processed in accordance with *Method I* are recorded in Table II and a typical

TABLE II.—ANALYSIS OF CODEINE PHOSPHATE IN PRESENCE OF ACETYLSALICYLIC ACID, PHENACETIN, AND CAFFEINE BY METHOD I

Mg. Taken	Mg. Recovered
27.76	28.16
36.66	36.65
39.26	39.35
34.28	34.38
36.93	36.86
Mean Recovery, %	100.35
Standard Deviation, %	±0.64

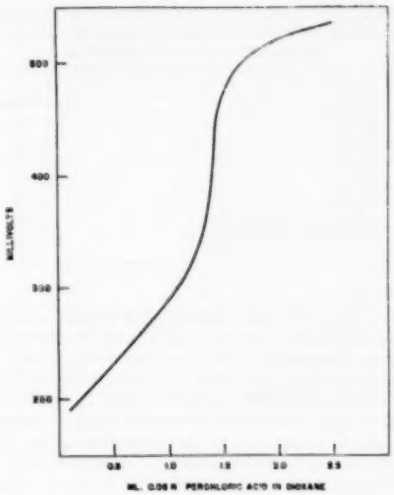

FIG. 2. TITRATION OF CODEINE PHOSPHATE IN PRESENCE OF ACETYLSALICYLIC ACID, PHENACETIN AND CAFFEINE USING A PHENOL – CHLOROFORM – ACETONITRILE SOLVENT SYSTEM.

titration curve is shown in Fig. 2. It will be noted that this curve exhibits almost the same slope as shown in Fig. 1. This indicates that titration of codeine phosphate in this solvent system is practically unaffected by the presence of the three other components, which are extracted from the sample along with the alkaloidal salt. The standard deviation was found to be 0.14% higher than that observed for similar assays on pure codeine phosphate, but this slight increase is probably due to the much smaller sample used for analysis rather than to the presence of additional ingredients. Figure 3 illustrates a typical titration curve obtained when acetylsalicylic acid, phenacetin, and caffeine are titrated with 0.05 *N* perchloric acid in dioxane. It will be noted that the only inflection is due to the small blank required for the solvent system.

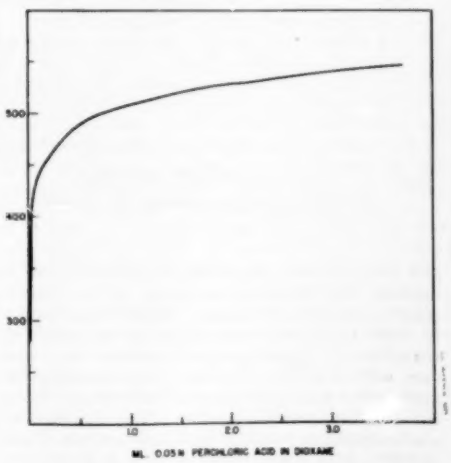

FIG. 3. TITRATION OF AN ACETYLSALICYLIC ACID, PHENACETIN AND CAFFEINE MIXTURE

TABLE III.—ANALYSIS OF CODEINE PHOSPHATE IN PRESENCE OF ACETYLSALICYLIC ACID, PHENACETIN, AND CAFFEINE BY METHOD II

Mg. Taken	Mg. Recovered
32.60	32.65
33.58	33.06
35.46	34.71
35.10	34.71
33.45	33.06
Mean Recovery, %	98.84
Standard Deviation, %	±0.84

In Table III are reported the results obtained when analyzing synthetic APC and C mixtures of known composition in accordance with *Method II*. This procedure is presented as an alternate to *Method I* and although more involved, offers to the analyst a procedure that insures removal of most interfering substances and is at the same time as accurate as the method first mentioned. Complete removal of the alkaloid was checked with Fulton's reagent for codeine (3). Again, to some of the samples variable amounts of the classical tablet excipients had been added prior to analysis. A typical titration curve is shown in Fig. 4. Because it is the free base

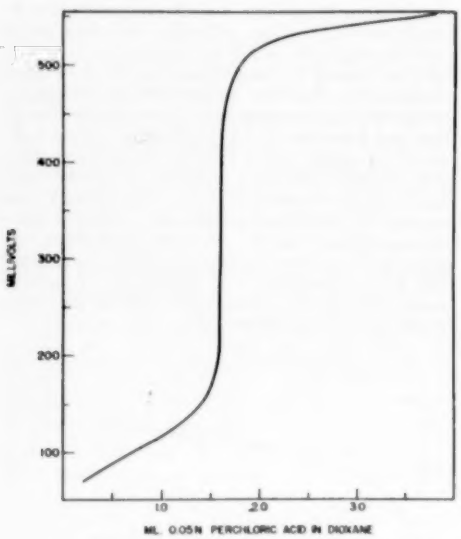

FIG. 4. TITRATION OF CODEINE EXTRACTED FROM A SYNTHETIC A.P.C. AND C TABLET

and not the alkaloidal salt which is titrated by this method, the potential change at the equivalence point is considerably greater (dE/dV was found to be 8,000 to 10,000) than that observed during the titration of analogous samples processed in accordance with *Method I*. The end point was therefore approached with increments of 0.01 ml. of titrant when following this procedure. The standard deviation based on five determinations was found to be ±0.84%, i. e., about 0.20% higher than that observed on comparable samples analyzed in accordance with *Method I*, a phenomenon evidently due to the chloroform extraction step introduced into the procedure.

The applicability of the method to commercial APC and C preparations as marketed in Canada is shown in Table IV. Seven of the products included

TABLE IV.—RECOVERY OF CODEINE PHOSPHATE FROM COMMERCIAL APC AND C TABLETS

Manufacturer	Labeled per Tablet, Mg.	Recovered per Tablet, Mg. *Method I*	*Method II*
A	32.40	33.18	32.94
		33.44	
B	32.40	30.41	30.03
		30.05	
C	32.40	34.20	
		33.69	
D	32.40	31.93	[a]
		31.61	
E	32.40	29.35	29.74
		29.61	
F	32.40	[a]	28.53
			28.98
J	32.40	33.65	
		33.19	
B	16.20	14.00	14.70
		14.25	
A	16.20	16.24	16.13
		16.31	
G	16.20	[a]	17.10
			16.84
E	16.20	[a]	14.50
			14.42
H	16.20	[a]	16.60
			16.21
I	16.20	15.33	15.51
		15.53	
A	8.10	7.91	
		8.07	
D	8.00	7.91	7.89
		7.94	
D	8.00	[a]	7.97
			7.91
G	8.10	[a]	8.31
			8.45
H	8.10	[a]	7.15
			7.11
B	8.10	7.76	7.78
		7.76	

[a] Interfering substances.

in this survey could not be accurately analyzed by *Method I*. Good recoveries, however, were always obtained by *Method II*, although in one case heavy emulsification during the extraction prevented recovery of the codeine. In the analysis of some of these commercial samples by *Method II*, five 20-ml. portions of chloroform were insufficient to extract the alkaloid. Therefore, in those samples where slight emulsification occurs, good pharmaceutical technique is necessary to remove the codeine completely. Satisfactory agreement is seen to exist when both methods of analysis were applied. Two of the preparations, containing salicylamide as an active ingredient, were successfully analyzed by either procedure. This indicates that the amide nitrogen does not affect the titration. Figure 5 shows a typical titration curve for a commercial tablet.

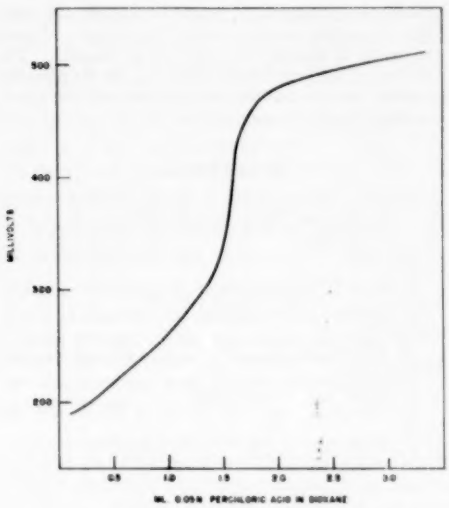

FIG 5. TITRATION OF A COMMERCIAL APC AND C TABLET

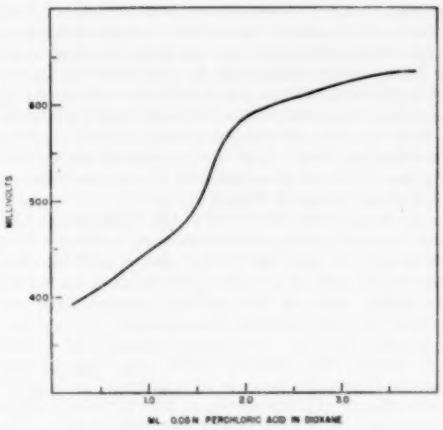

FIG 7. TITRATION OF CODEINE PHOSPHATE IN PRESENCE OF ACETYLSALICYLIC ACID, PHENACETIN AND CAFFEINE USING A GLACIAL ACETIC ACID SOLVENT SYSTEM

DISCUSSION

The lack of adequate procedures for assaying APC and C preparations for codeine phosphate stems primarily from the presence of caffeine. Being also a nitrogenous base, this compound tends to interfere with the quantitative separation and estimation of the opium alkaloid. Thus, using glacial acetic acid as solvent, pure codeine phosphate may be readily determined by potentiometric titration with perchloric acid (Fig. 6), but no satisfactory potentiometric break occurs in the titration when this technique is applied to codeine phosphate-caffeine mixtures or to APC and C combinations (Fig. 7). Similarly, pure caffeine can be accurately determined by titration with perchloric acid in acetic anhydride-nitromethane systems (4) but again mixtures of codeine phosphate and caffeine cannot be assayed by this method (5).

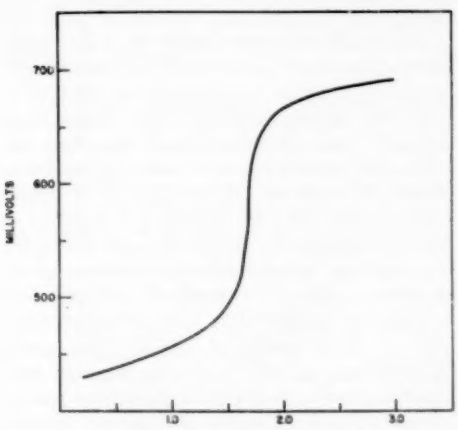

FIG 6. TITRATION OF CODEINE PHOSPHATE IN GLACIAL ACETIC ACID

A number of differential nonaqueous titrations have been described in the literature. Thus Fritz was able to analyze aromatic-aliphatic amines by titration with perchloric acid (6). Pifer, Wollish, and Schmall showed how certain pairs of inorganic cations may be determined in nonaqueous systems by this technique (7). Wagner described a nonaqueous method for tertiary aliphatic amines in the presence of primary and secondary amines (8). The main requirement for carrying out such assays is a solvent system, in which the pK values of the compounds to be determined lie sufficiently far apart to permit performance of a differential analysis. The authors found that phenol, chloroform, and acetonitrile, in the proper proportions, fulfilled this requirement. In this system caffeine exhibits very weakly alkaline properties, whereas the opium alkaloid behaves as a strong base and can be titrated directly with perchloric acid in dioxane

Phenol-chloroform mixtures were originally used as solvent media by Dietzel and Paul (9) for the aminometric titration of morphine. They postulated that the alkaloid, which is only slightly soluble in chloroform, undergoes complex formation with phenol and that the alkaloidal complex generated solubilizes readily in chloroform. Codeine phosphate apparently behaves in a similar fashion, for, although highly insoluble in chloroform (1:5,000), it was found to dissolve quite easily in the presence of phenol. The binary solvent mixture was not suitable, however, for the potentiometric titration of the alkaloidal salt with perchloric acid in either glacial acetic acid or dioxane. Galvanometer readings were extremely erratic, particularly in the neighborhood of the equivalence point and no satisfactory titration curve could be obtained. Upon addition of acetonitrile to the solvent system and the use of a solution of perchloric acid in dioxane, reaction conditions were sufficiently stabilized to insure sensitive operation of the apparatus.

A blank of 0.01 to 0.02 ml. of the acid was required for the solvent system and applied as a cor-

rection factor. Although a total volume of only about 1.6 ml. of the titrant was used per determination, satisfactory accuracy was achieved in all cases.

Most tablet excipients do not interfere in the determination of the alkaloidal salt. However, it was found that magnesium stearate, which is slightly soluble in the phenol-chloroform solvent system, resulted in about 1% overestimation of the codeine content. Where it is present, it can easily be removed by the use of *Method II*.

In the analysis of several of the commercial APC and C preparations, recoveries were much less than expected. It was found that the interfering substance was gelatin. It is postulated that during the tableting some of the codeine phosphate is adsorbed onto the surface of the gelatin. It is generally accepted (10) that when solutions of gelatin are cooled, the particles form into long threads which eventually become interlocked. It is probably due to this phenomenon and to some surface adsorption that a portion of the codeine phosphate is not recovered in the procedure. The phenol-chloroform mixture is probably unable to solubilize this fraction of the codeine phosphate. Again, the use of *Method II* gave satisfactory results. The addition of water to the powdered tablet reverses the gelation process and makes the codeine phosphate available for the determination.

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Isotonic Solutions*

IV. Urea and Urea Derivatives

By WILLIAM D. EASTERLY, Jr.,† and WILLIAM J. HUSA‡

The purpose of the present investigation was to obtain data by the hemolytic method that might be of value in the preparation of isotonic solutions, since other methods generally used are based on assumptions often giving erroneous results. Experiments were conducted to determine what effect urea and various urea derivatives have in preventing hemolysis of rabbit and human erythrocytes.

THE HEMOLYTIC method has been used extensively in permeability studies. However, little application of the method has been made in the study and preparation of isotonic solutions. Husa and Adams (1) showed that the hemolysis of red blood cells is not prevented by certain substances at concentrations calculated to be isotonic according to osmotic pressure based on colligative properties; they pointed out the necessity of making hemolytic tests in order to determine whether data obtained by methods based on colligative properties would be applicable to the preparation of parenteral solutions. Husa and Grosicki (2) made a quantitative study of the hemolytic method in the determination of sodium chloride equivalents of various substances.

In the present study quantitative determinations were made of the degree of hemolysis of rabbit and human erythrocytes in solutions of urea and various urea derivatives. Experiments were also conducted to determine the effect that urea and urea derivatives have in preventing hemolysis when used in the presence of 0.4 per cent sodium chloride. Hober (3) pointed out that in solutions of "indifferent" nonelectrolytes, erythrocytes undergo severe alterations which, however, could be reduced by adding small amounts of an electrolyte, such as sodium chloride. A concentration of 0.4 per cent sodium chloride was chosen for testing purposes since that concentration is almost enough to prevent hemolysis of both rabbit and human erythrocytes; thus any added effect produced by an added substance could easily be detected.

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This paper is based on a dissertation presented to the Graduate Council of the University of Florida by William D. Easterly, Jr., in partial fulfillment of the requirements for the degree of Doctor of Philosophy.
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‡ Head, Department of Pharmacy, University of Florida.

Other determinations were made in the presence of 0.6 per cent sodium chloride. The purpose of such experiments was to find whether any of the derivatives increased erythrocyte fragility to the extent of allowing hemolysis even when the salt concentration was sufficient to prevent such hemolysis if no other substance were present. Wokes (4) reported that boric acid acted in such a way, as it was hemolytic in all concentrations.

EXPERIMENTAL

Collection of Blood.—The rabbit blood used in the various experiments was obtained by heart puncture. The blood was immediately defibrinated by gentle rotation in a flask with glass beads until the fibrin had separated. The defibrinated blood was then poured into a small flask and aerated by gently swirling the flask for approximately five minutes. The human blood used in the course of the experiments was obtained from the veins of the arm of a twenty-seven year old male and a twenty-five year old female by means of a hypodermic syringe. It was treated in exactly the same manner as the rabbit blood. Each experiment was repeated using a fresh sample of rabbit or human blood.

Preparation of Solutions.—For each urea derivative tested the concentration having the same osmotic pressure as a 0.54% sodium chloride solution was calculated by the osmotic factor method using 1.86 as the value of i (isotonic coefficient) for sodium chloride, and 1.00 as the value of i for the urea derivative. Since the calculations indicated that for each derivative the concentration necessary to produce the isotonic solution fell between 1 and 2%, stock solutions ranging from 1 to 6% were prepared for testing. All of these solutions were prepared by bringing to volume in a volumetric flask with triple distilled water.

To prepare the solutions containing both sodium chloride and a urea derivative, the proper amounts of 10% solutions for each to give the desired concentration were pipetted into volumetric flasks. These were then brought to volume with triple distilled water. In a few instances, due to low solubility of certain derivatives, stock solutions of less than 10% were used.

Quantitative Determination of Per Cent Hemolysis.—The method used to determine the degree of hemolysis of erythrocytes in the various solutions was essentially that of Hunter (5). It is a method based on the fact that the oxyhemoglobin liberated by a hypotonic salt solution is directly proportional to the number of cells hemolyzed. Complete hemolysis is obtained by laking the erythrocytes in a 0.1% solution of sodium carbonate; therefore, the hemolysis occurring in a given solution can be expressed in terms of total hemolysis. A quantitative determination of hemolysis in various solutions is then made by centrifuging off the unhemolyzed cells and establishing the amount of oxyhemoglobin in the supernatant fluid by means of a photoelectric colorimeter. Details of the hemolytic method have been given by Husa and Grosicki (2).

Data.—Quantitative determinations were made of the degree of hemolysis of rabbit and human erythrocytes in 1 to 6% solutions of urea and various urea derivatives. The results of these determinations are not tabulated since approximately 100% hemolysis occurred in each solution tested. Tables are presented for both rabbit and human blood to show the effect that urea and the urea derivatives have in preventing hemolysis when used in the presence of 0.4% sodium chloride. Other tables give the results of experiments made to determine whether the substances increase erythrocyte fragility to the extent of hemolysis even in the presence of 0.6% sodium chloride. The figures for per cent hemolysis as shown in the tables are an average of the results obtained with two different blood samples.

DISCUSSION

Urea.—Quantitative determinations indicated that approximately 100% hemolysis of rabbit and human erythrocytes occurred in urea solutions of 1.0 to 6.0%. According to osmotic calculations, a 1.04% urea solution would have the same osmotic pressure as a 0.54% solution of sodium chloride, and a urea solution of that particular concentration would generally be considered sufficient to prevent the hemolysis of rabbit and human erythrocytes. Since this is not the case, however, it can readily be seen that concentrations of urea calculated by colligative properties to be isosmotic with blood are not necessarily isotonic in those proportions. Furthermore, concentrations of urea much higher than that calculated to be isosmotic were shown to have no effect in preventing hemolysis.

Since tonicity is determined by the concentration of solute particles that cannot pass through the cell membrane rather than by the total concentration of solute particles in solution, urea appears to penetrate the erythrocyte in all concentrations tested. This assumption agrees with the findings of previous investigators, including Grijns (6), Hedin (7) and Jacobs (8).

Table I indicates that urea had little or no effect in preventing hemolysis of rabbit erythrocytes in solutions containing 0.4% sodium chloride and various concentrations of urea. Approximately the same per cent of hemolysis occurred in the solutions containing urea with sodium chloride as when the same concentration of sodium chloride was used alone. As indicated by Table II, in solutions containing 0.6% sodium chloride and concentrations of urea ranging from 0.1 to 5.0%, no hemolysis occurred; this did not parallel the findings of Ebina (9), who reported that urea in concentrations of 0.5 to 6.0% caused hemolysis independently of the concentration of sodium chloride.

Urea was similarly shown to have little effect in preventing hemolysis of human blood and appeared to increase erythrocyte fragility after the concentration reached a certain point (Table III). For instance, in solutions containing 0.4% sodium chloride and 3.0 to 5.0% urea, hemolysis was greater than in solutions containing only 0.4% sodium chloride. The lower concentrations of urea with 0.4% sodium chloride either showed no effect or gave a slight lowering of the degree of hemolysis. There was no hemolysis of human erythrocytes in solutions containing 0.6% sodium chloride and varying proportions of urea (Table IV).

TABLE I.—HEMOLYSIS AT 25° C. OF RABBIT ERYTHROCYTES IN SOLUTIONS CONTAINING 0.4 PER CENT SODIUM CHLORIDE AND VARIOUS PROPORTIONS OF A UREA DERIVATIVE

Urea Derivative, %	NaCl, %	Urea	Thiourea	Urethan	n-Propyl Carbamate	Methylurea	1,3-Dimethylurea	Ethylurea
0.0	0.4	94.6	95.3	94.4	95.7	68.6	98.1	73.4
0.1	0.4	89.0	91.5	92.4	93.5	68.4	91.9	64.6
0.3	0.4	89.2	91.9	93.4	93.7	65.5	94.0	64.2
0.5	0.4	92.1	90.3	91.7	95.6	62.4	95.2	64.5
1.0	0.4	94.6	92.3	93.0	95.4	66.3	97.4	66.2
3.0	0.4	96.6	99.4	93.1	96.4	65.4	95.9	75.5
5.0	0.4	100.2	100.0	90.6	100.0	54.5	99.9	87.6

TABLE II.—HEMOLYSIS AT 25° OF RABBIT ERYTHROCYTES IN SOLUTIONS CONTAINING 0.6 PER CENT SODIUM CHLORIDE AND VARIOUS PROPORTIONS OF A UREA DERIVATIVE

Urea Derivative, %	NaCl, %	Urea	Thiourea	Urethan	n-Propyl Carbamate	Methylurea	1,3-Dimethylurea	Ethylurea
0.0	0.6	0.5	0.0	0.6	1.4	0.0	0.0	0.0
0.1	0.6	0.8	0.0	2.7	0.6	0.0	0.0	0.0
0.3	0.6	0.0	0.0	1.6	1.2	0.0	0.0	0.4
0.5	0.6	0.0	0.0	1.0	0.5	0.0	0.0	1.4
1.0	0.6	1.8	1.2	3.4	0.8	0.0	0.0	1.0
3.0	0.6	0.3	9.1	2.0	0.0	0.0	0.0	1.1
5.0	0.6	0.6	10.3	2.1	100.0	0.0	1.6	1.0

Urea Derivatives.—Thiourea had little or no effect in preventing hemolysis of rabbit erythrocytes even in the presence of 0.4% sodium chloride. Furthermore, concentrations of 1.0 to 5.0% thiourea appeared to increase erythrocyte fragility, causing a slight degree of hemolysis in solutions containing 0.6% sodium chloride (Table II). This effect was more pronounced than with urea in the same contrations. With human blood, it appeared that 0.1 to 0.5% thiourea with 0.4% sodium chloride had some effect in preventing hemolysis (Table III). However, as with rabbit blood, 1.0 to 5.0% thiourea with 0.4% sodium chloride increased the degree of hemolysis over that in solutions of 0.4% sodium chloride alone. As shown in Table IV, a slight degree of hemolysis of human erythrocytes occurred in solutions of 3.0 to 5.0% thiourea with 0.6% sodium chloride, thus again indicating that higher concentrations of thiourea increased erythrocyte fragility.

Examination of Table I reveals that urethan had little effect in preventing hemolysis of rabbit erythrocytes in the presence of 0.4% sodium chloride. Erythrocyte fragility, however, did not appear to

TABLE III.—HEMOLYSIS AT 25° C. OF HUMAN ERYTHROCYTES IN SOLUTIONS CONTAINING 0.4 PER CENT SODIUM CHLORIDE AND VARIOUS PROPORTIONS OF A UREA DERIVATIVE

Urea Derivative, %	NaCl, %	Urea	Thiourea	Urethan	n-Propyl Carbamate	Methylurea	1,3-Dimethylurea
0.0	0.4	34.2	29.8	29.1	24.5	32.6	39.5
0.1	0.4	29.8	14.1	19.5	14.7	21.3	24.0
0.3	0.4	30.7	15.5	18.2	11.4	23.7	26.4
0.5	0.4	30.5	17.2	16.7	9.2	21.4	23.0
1.0	0.4	33.6	37.0	16.1	7.9	27.3	29.2
3.0	0.4	48.7	52.6	13.3	37.2	16.0	35.2
5.0	0.4	74.8	67.7	15.5	98.3	8.3	48.8

TABLE IV.—HEMOLYSIS AT 25° C. OF HUMAN ERYTHROCYTES IN SOLUTIONS CONTAINING 0.6 PER CENT SODIUM CHLORIDE AND VARIOUS PROPORTIONS OF A UREA DERIVATIVE

Urea Derivative, %	NaCl, %	Urea	Thiourea	Urethan	n-Propyl Carbamate	Methylurea	1,3-Dimethylurea
0.0	0.6	0.0	0.0	0.0	0.0	0.0	0.0
0.1	0.6	0.0	0.0	0.4	0.0	0.0	0.0
0.3	0.6	0.0	0.0	0.0	0.0	0.0	0.0
0.5	0.6	0.9	0.0	0.4	1.1	0.5	0.0
1.0	0.6	0.0	0.0	0.3	2.5	0.4	0.0
3.0	0.6	0.0	1.0	1.3	1.6	1.0	0.0
5.0	0.6	0.0	2.2	0.9	100.0	0.4	0.0

increase to any appreciable extent as the concentration of urethan was increased and the sodium chloride concentration kept constant. From Table II, it can be seen that urethan may have exhibited a slight effect in preventing hemolysis of human erythrocytes in the presence of 0.4% sodium chloride, and that the higher concentrations did not appear to increase erythrocyte fragility. Only a slight degree of hemolysis occurred in any of the urethan solutions containing 0.6% sodium chloride (Tables II and IV).

From Table I, it can be seen that *n*-propyl carbamate also had little effect in preventing hemolysis of rabbit erythrocytes in the presence of 0.4% sodium chloride. From Table III, it may be noted that concentrations of 0.1 to 1.0% *n*-propyl carbamate with 0.4% sodium chloride had some effect in preventing hemolysis of human blood, the greatest effect being shown with 1.0% *n*-propyl carbamate. Higher concentrations of *n*-propyl carbamate increased the degree of hemolysis. As shown in Tables II and IV, a concentration of 5.0% *n*-propyl carbamate caused 100% hemolysis of both human and rabbit erythrocytes even in the presence of 0.6% sodium chloride. Concentrations of *n*-propyl carbamate lower than 5.0% did not appear to affect erythrocyte fragility to any appreciable extent.

Methylurea seemed to have some effect in the prevention of hemolysis of both rabbit and human erythrocytes when used in the presence of 0.4% sodium chloride. As the concentration of methylurea was increased from 0.1 to 5.0% the degree of hemolysis decreased. Four rabbit blood samples and four human blood samples were used to verify the results since the effect mentioned did not hold true for the other urea derivatives tested. The hemolysis occurring in methylurea solutions containing 0.6% sodium chloride was negligible.

Table I shows that 1,3-dimethylurea contributed very little toward the prevention of hemolysis of rabbit erythrocytes in solutions containing 0.4% sodium chloride. Concentrations of 1,3-dimethylurea up to 3.0% had some effect in preventing hemolysis of human erythrocytes when used with 0.4% sodium chloride; higher concentrations, however, caused an increase in hemolysis (Table III). No concentration of 1,3-dimethylurea tested caused any appreciable hemolysis of either rabbit or human erythrocytes when 0.6% sodium chloride was present (Tables II and IV).

Concentrations of ethylurea up to 1.0% exhibited a slight effect toward lowering the hemolysis of rabbit erythrocytes in solutions containing 0.4% sodium chloride; concentrations above 1.0% caused an increase in hemolysis. Only a slight degree of hemolysis occurred in any of the solutions containing 0.6% sodium chloride.

Difference in Results with Rabbit Blood and Human Blood.—The changes in hemolysis were more pronounced for human blood than for rabbit blood when tested with solutions of a urea derivative with 0.4% sodium chloride. This difference in results can probably be explained by the fact that rabbit erythrocytes begin to hemolyze in sodium chloride solutions when the concentration falls below 0.54, while human erythrocytes remain intact until the sodium chloride concentration drops to approximately 0.44%. Thus, a 0.4% solution of sodium chloride allows only partial hemolysis of human erythrocytes, while most rabbit blood samples show almost complete hemolysis in this concentration of sodium chloride. Any increased hemolytic action, therefore, could not be detected with rabbit erythrocytes.

Structure and Activity.—Urea contributed very little to the prevention of hemolysis; even in the presence of 0.4% sodium chloride no preventive effect could be detected with rabbit blood, and only a slight effect with human blood was noted. The substitution of a methyl group for one hydrogen (methylurea) seemed to contribute to the prevention of hemolysis, but when the substituent group was ethyl no preventive effect was detected. A second methyl group as in 1,3-dimethylurea caused a loss of the property of contributing to the prevention of hemolysis.

Urethan and *n*-propyl carbamate, whose structures differed by only one methyl group, were very different in their action; *n*-propyl carbamate in a 5.0% concentration caused 100% hemolysis of both rabbit and human erythrocytes even in the presence of 0.6% sodium chloride, but urethan showed no effect when tested in the same way.

Thiourea gave much the same results as urea.

SUMMARY

1. Quantitative determinations indicated that approximately 100 per cent hemolysis of rabbit and human erythrocytes occurred in 1.0 to 6.0 solutions of urea and all the urea derivatives tested. According to osmotic calculations, the concentration of each substance necessary to produce an isotonic solution would be between 1.0 and 2.0 per cent, but it was found that concentrations much higher than this had no effect in preventing hemolysis.

2. Experiments were made to determine the effect that urea and the urea derivatives have in preventing hemolysis in the presence of 0.4 per cent sodium chloride. The greatest effect was shown by methylurea which apparently caused a decrease in hemolysis of both rabbit and human erythrocytes in concentrations of 0.1 to 5.0 per cent. As a rule, the other derivatives showed little effect in preventing hemolysis of rabbit erythrocytes even when 0.4 per cent sodium chloride was present; with human blood, however, a slight decrease with low concentrations and an increase with high concentrations could be detected.

3. When tested with 0.6 per cent sodium chloride, only *n*-propyl carbamate appeared to cause any appreciable increase in hemolysis of erythrocytes. In a solution of 5.0 per cent *n*-propyl carbamate with 0.6 per cent sodium chloride, 100 per cent hemolysis of both human and rabbit erythrocytes occurred; lower concentrations of *n*-propyl carbamate did not show this effect, however.

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The Formulation of Tablet Implants Using Polyethylene Glycol 6000 and Methylcellulose*

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A quantitative method is described for the evaluation of tablet implants. By use of a dye that is completely excreted from the system, the relative efficiency of different implant bases, Carbowax® 6000, mixture of Carbowax® and Methocel® 4000, pharmagel B, cholesterol, Sterotex® and Methocel® 4000 has been determined. Cholesterol and Sterotex® were not absorbed even after eight weeks. Cholesterol excreted dye in a thirty-hour period whereas Sterotex® did not release at all. Pharmagel B can be satisfactorily used as a tablet implant meant to last not longer than seventy-two hours by itself. However, pharmagel tablets are difficult to make. Carbowax® 6000 can be satisfactorily used as a short-period implant not lasting longer than thirty-six to forty-eight hours by itself and releasing the highly water-soluble drug in six hours. When mixed with Methocel® in different proportions, the rate of release can be controlled somewhat. Pure Methocel® 4000 can be used satisfactorily as a base. It is itself absorbed in seventeen to nineteen days and releases the dye in thirty hours. However, difficulty was observed in making tablets. This difficulty was due to the fluffiness and low density of Methocel® 4000.

THE USE of implanting drugs subcutaneously for the administration of sex hormones was demonstrated to be a practical method of hormone therapy by Deansely and Parkes (1) in 1937. These workers implanted compressed pellets of the pure hormone and found that the physiological action was continuous, intense, and prolonged. The availability of numerous hormones and their esters with wide ranges of absorbability and potency permitted them to predict that "additional information should thus make possible treatment of almost any intensity and duration by a single administration" of steroid hormones in this form. The technique used by Deansely and Parkes was as follows:

Tablets for implantation were made by compression of the undiluted crystalline hormone in a micropastille press. The animals were anesthetized with ether and weighed tablets were implanted under the skin through a small slit which afterward was closed by a stitch. The animal was sacrificed and the tablet removed. Finally the tablet was dried in a desiccator and weighed.

Pellets of undiluted hormones have since found wide usage in medical practice. However the administration of the undiluted hormone is not always desirable because of such facts as rate of absorption and the development of undesirable tissue at the site of implantation. Several workers have therefore experimented with modification of the composition of the tablet implant as well as chemical modifications of the chemical structure of the medicinally active agent. Kohn and Bulger (2) in 1937 showed that zinc sulfate, a protein precipitant, retarded the absorption of adrenalin when this drug was injected subcutaneously. Deansely and Parkes (1) studied the effects of esterification of testosterone and androsterone on the rate of absorption of each of these hormones. It was shown by these workers that esterification of hormones caused a delayed absorption. Parkes (3) experimented with such agents as stearic acid, palmitic acid, lumisterol, ergosterol, sitosterol, uric acid, starch, and kaolin as vehicles for hormones in an attempt to reduce the rate of absorption. His experiments indi-

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cated that these agents did not affect the rate of absorption of the hormone. Parkes (3) also worked with cholesterol, cholesterol with dextrose, thyroxine, and benzylsulfanilamide, and found that these substances did have some effect on the rate of absorption. Green and January (4) made pellets containing beeswax or lanolin but found that these substances exerted no effect on absorption rate.

From this brief review of the development of tablet implants it is evident that the pharmaceutical knowledge of the nature of tablet implants is limited and that investigations into the nature of tablet implants would be of great value. Furthermore, since the work of Deansely and Parkes, Kohn and Bulger, and of Green and January, new synthetic products have become available which indicate by their physical and chemical properties that they might be suitable bases for tablet implants. It is with these new products in mind that the present investigation was made.

The essentials of a good implant are: (*a*) The raw materials should be pure and easily compressible. (*b*) The implant should not produce local necrosis; irritation, inflammation, or tenderness. (*c*) The implant should dissolve slowly in the cellular fluids and blood stream. (*d*) The rate of solution should be even and easily controllable. (*e*) Its use should not result in the formation of a ghost or fibrous capsule.

Applying these critieria to the technique used and results achieved by Deansely and Parkes (1), it can be seen that their investigations had several shortcomings. First of all, they did not determine the time rquired for complete absorption of the bases. Second, water-soluble bases were not used. Third, the tablets were taken out and reweighed, the absorption being determined by loss in weight; but this weight loss was so small as to have possibly resulted from experimental error. The same shortcomings found in the Deansely and Parkes experiment inhere in the studies of Deansely working alone, Noble and Parkes working alone, as well as in the investigations of most of those workers who have since dealt with this problem. In every case the technique used has been that of the 1927 Deansely and Parkes experiment previously described.

EXPERIMENTAL

Carbowax® 6000.—Since polyethylene glycols have found application as a lubricant in tablet manufacture (5), it was felt that it would be possible to use them in the formulation of a base for tablet implants. In order to test their possibilities the experiment described below has been conducted.

Carbowax® 6000, being of higher molecular weight and less soluble in water, was selected for the primary study. Carbowax® 6000 passed through a No. 20 sieve and then through a No. 40 sieve. This powder was then sterilized by spreading it on paper in thin layers and then exposing it to ultraviolet light.

The No. 40 powder was then compressed into tablets on a Eureka tablet machine equipped with a $^7/_{32}$-inch punch and die. The machine base, platform, punch, and die were all sterilized with 70% alcohol.

Two groups of five albino rats weighing from 120–160 Gm. each, were anesthetized with ether, and previously weighed tablets of Carbowax® 6000 were implanted $^1/_4$–$^1/_2$ inch behind the ventral side of the neck portion. It was found from this primary study that the Carbowax® 6000 tablets were completely absorbed in the blood stream in 36–48 hours. It is worth while to note that there was no fibrous capsule formed.

Methylcellulose.—Methocel® is widely used, and with excellent results, as an adhesive in agriculture, a binder in tablet manufacture and burn therapy, and as both adhesive and binder in the ceramics, soap and cosmetics, and leather industries. Its wide usage and slow solubility in water led to the thought that it would be possible to use it as a base for tablet implants.

The Methocel® 4000 tablets were made as follows:

Methocel® 4000	100 Gm.
Water, dist	100 cc.

Methocel® 4000 powder was moistened with water and the wet mass was then passed through a No. 20 sieve. The resulting granules were dried at 40° for three hours. The dried granules were then passed through a No. 40 sieve and sterilized as before. The sterilized granules were compressed into about 100 mg. tablets in a Eureka machine equipped with a $^7/_{32}$-inch punch and die.

These tablets were implanted into two batches of five rats as before. It took 17 to 19 days for the complete absorption of the implant. This led to the thought that it may be possible to make implants which can dissolve or be absorbed in anywhere from 2 to 17 days by taking implants containing different amounts of Carbowax® and Methocel.®

The method of making tablets containing different amounts of Carbowax® 6000 and Methocel® 4000 is as follows:

I.	Carbowax® 6000	50 Gm.
	Methocel® 4000	50 Gm.
	Water, dist	30 cc.
II.	Carbowax® 6000	33.3 Gm.
	Methocel® 4000	66.6 Gm.
	Water, dist	30 cc.
III.	Carbowax® 6000	25 Gm.
	Methocel® 4000	75 Gm.
	Water, dist	40 cc.

In all the three formulas given above, Carbowax® 6000 and Methocel® 4000 were mixed thoroughly by trituration in a mortar. The mixture was granulated with water and the wet mass pressed through a No. 20 Sieve. The mixture was dried in air and the dry granules passed through a No. 40 sieve. The granules were then sterilized and compressed into about 100-mg. tablets.

The implantation of these tablets took 9–10, 13–14, and 15 days, respectively, for complete absorption.

Gelatin (Pharmagel B).—Pharmagels are slowly soluble in water. Their wide pharmaceutical applications suggested that they might prove of value as an implant base.

Pharmagel B is obtained in coarse powder form and this powder is hard to compress. It was found that it can be better compressed if the coarse powder is dampened with alcohol. Thirty cubic centimeters of alcohol was used per every 100 Gm. of pharmagel B. Tablets weighing 100 mg. were made.

When implanted in two batches of five rats these tablets showed complete absorption in seventy-two hours.

Cholesterol.—An animal sterol, cholesterol can act as an implant base without any serious shortcomings. This base is slightly soluble in water as well as in alcohol. Parkes (3) in 1942 showed that cholesterol was a very satisfactory base for delaying the absorption of water-soluble substances.

Cholesterol tablets were made as follows:

Cholesterol	100 Gm.
Alcohol	60 cc.

Cholesterol was granulated by alcohol and the mass was pressed through a No. 40 sieve. These granules were then dried at 120° F., sterilized as before, and compressed into about 100-mg. tablets.

Even after two months of implantation these tablets showed no appreciable loss in weight and no change whatsoever in shape.

Sterotex®[1]	100 Gm.
Alcohol	100 cc.

Sterotex® was granulated with alcohol. The wet mass was pressed through a No. 40 sieve and dried in air. These granules were then compressed into about 100-mg. tablets.

Implantation of these tablets showed that there was no absorption even after eight weeks.

The results of the implantation of blank tablets are presented in Table I.

TABLE I.—RESULTS OF IMPLANTATION OF BLANK TABLETS

	Type of Implant	Time for Complete Absorption
1.	Carbowax® 6000	36–48 hours
2.	Pharmagel B	72 hours
3.	Carbowax® 6000 + Methocel® 4000 (1:1)	9–10 days
4.	Carbowax® 6000 + Methocel® 4000 (1:2)	13–14 days
5.	Carbowax® 6000 + Methocel® 4000 (1:3)	15 days
6.	Methocel® 4000	17–19 days
7.	Cholesterol	No absorption even after 8 weeks
8.	Sterotex®	No absorption even after 8 weeks

[1] Capital City Products, Columbus, Ohio (a hydrogenated fat).

METHOD OF EVALUATION

In view of the favorable results of the preliminary experiments, it was felt necessary to devise some method by which the efficiency of the bases for implants can be evaluated.

Different investigators in the field tried the bases which were insoluble in water. They tried to find the decrease in weight of base by taking the base out from the site of implantation, then washing, drying, and weighing the base. This method has many disadvantages, such as loss during implanting as well as during taking out, washing, and drying. It was observed that the loss in weight of the tablet was so small that it was very difficult to say whether this loss was due to experimental error.

Phenolsulfonphthalein (P. S. P. or phenol red) was used as a water-soluble indicator system. The sodium salt of the phenolsulfonphthalein is soluble in water and alcohol and is completely excreted from the body. The rate of release of this dye can easily be measured by adding 25% 0.1 *N* sodium hydroxide of the total volume. Thus the method of evaluation was based on its excretion in the urine from the implanted rats. One per cent phenolsulfonphthalein sodium tablets using different bases were made and with sterile technique implanted in the rat. Then the rat was placed in its individual, specially designed metabolic cage. The concentration of the phenolsulfonphthalein sodium excreted in the urine collected every six hours was determined colorimetrically on the basis of the change in color of phenolsulfonphthalein from yellow in acid media to pink in alkaline media.

PREPARATION OF TABLETS

The initial step in the preparation of the tablets involves the conversion of the ingredients from a finely powdered or from a large crystalline form to a granulated form. A conventional method of granulation, referred to as the moist granulating process, consists of the addition to the powder mixture of moistening liquids, such as alcohol, water, or a mixture of the two, with a binder dissolved therein, which causes fine particles to adhere and to remain adherent after drying. Binder solutions commonly used in tablet making were not used, since they might exert some effect on rate of release of the drug. Many bases used in the experiment were binders themselves, or had some binder effect.

An alternative method of granulation is that of precompression, or slugging. The mixed ingredients in finely powdered form are compressed into slugs or disks, which are then broken and passed through a sieve of proper size. Unsatisfactory results were obtained in the case of pharmagel B, which possesses poor compression qualities.

Granulation of finely divided materials may be effected by use of pure alcohol, water, or a mixture of the two. Sufficient solvent is used to dissolve phenolsulfonphthalein sodium and to convert the powder to a damp nonfluid mass. The moistened mass is forced through a sieve and dried. The size of the mesh used depends upon the size of the tablet to be made. In all cases, a No. 40 mesh was used and the compression of the granules was accomplished without a lubricant.

PROCEDURE

To standardize one of the variables—the size of the implant—the punch and die used was $^{7}/_{32}$ inch throughout the experimental work. As far as possible the pressure in making tablets was kept constant. The effect of compression on the rate of absorption is at the most small and, in practice, of no significance (6). The weights of all pellets ranged from 85-140 mg.

Preparation of Carbowax® 6000 Tablets with P. S. P.

Carbowax® 6000	100 Gm.
Phenolsulfonphthalein sodium	1.00 Gm.
Water, dist.	7.5 cc.

The phenolsulfonphthalein sodium was dissolved in 5 cc. of water and this solution was added to polyethylene glycol 6000 powder with trituration. Then the rest of the water was added and mixed well. After thorough incorporation, the damp mass was pressed through a No. 20 sieve and the resulting granules were dried in air. The granules were then passed through a No. 40 sieve.

In this and in the subsequent phases of the experiment the granules were pressed into tablets on a Eureka, single punch, hand-operated tablet machine, punches and die of $^{7}/_{32}$ inch being used to prepare about 100-mg. tablets.

These phenolsulfonphthalein sodium tablets were assayed by a Cenco-Sheard photelometer. In each case twenty tablets were powdered and an aliquot of 100 mg. was taken and dissolved in about 50 cc. of water and then diluted to 500 cc. after being made distinctly alkaline with 5 cc. of 0.1 *N* sodium hydroxide.

Phenolsulfonphthalein sodium tablets with Carbowax® 6000 base assayed at 0.9%.

Preparation of Phenolsulfonphthalein Sodium Tablets Using Carbowax® 6000 Base Diluted With Equal Amount of Methocel® 4000

Polyethylene glycol 6000	50 Gm.
Methocel® 4000	50 G.n.
Phenolsulfonphthalein sodium	1.00 Gm.
Water, dist.	30 cc.

Polyethylene glycol 6000 powder and methylcellulose 4000 were thoroughly triturated in a mortar and then moistened with the aqueous solution of phenolsulfonphthalein sodium. The wet mass was pressed through a No. 20 sieve and air dried. The dried granules were pressed through a No. 40 sieve and compressed into tablets of about 100 mg.

When assayed colorimetrically, these tablets gave 0.95% phenolsulfonphthalein sodium per tablet.

Preparation of Phenolsulfonphthalein Sodium Tablets Using Carbowax® 6000 Base Diluted with Twice the Amount of Methocel® 4000

Carbowax® 6000	33.3 Gm.
Methocel® 4000	66.6 Gm.
Phenolsulfonphthalein sodium	1.0 Gm.
Water, dist.	30 cc.

Carbowax® 6000 powder and Methocel® 4000 powder were thoroughly mixed by trituration and the mixture moistened with an aqueous solution of phenolsulfonphthalein sodium. The wet mass was then pressed through a No. 20 sieve and dried at 40° C. for three hours. The dried granules were pressed through a No. 40 sieve and compressed into about 100-mg. tablets.

The tablets were then assayed colorimetrically for P. S. P. as shown above. Each tablet contained 0.975 per cent phenolsulfonphthalein sodium.

Preparation of Phenolsulfonphthalein Sodium Tablets Using Carbowax® 6000 Base Diluted With Three Volumes of Methocel® 4000

Carbowax® 6000	25 Gm.
Methocel® 4000	75 Gm.
Water, dist.	40 cc.
Phenolsulfonphthalein sodium	1.00 Gm.

Carbowax® 6000 and Methocel® 4000 were mixed in powdered form and granulated with an aqueous solution of phenolsulfonphthalein. The wet mass was forced through a No. 20 sieve and the granules were dried at 40° C. for three hours. The dried granules were passed through a No. 40 sieve and compressed into about 100-mg. tablets.

These tablets when assayed in the usual manner were seen to contain 0.95% phenolsulfonphthalein sodium.

Preparation of Phenolsulfonphthalein Tablets Using Methocel® 4000 as a Base

Methocel® 4000 powder	100 Gm.
Phenolsulfonphthalein sodium	1.00 Gm.
Water, dist.	100 cc.

Methocel® powder was granulated with an aqueous solution of phenolsulfonphthalein sodium. The wet mass was then forced through a No. 20 sieve and the resulting granules were dried at 120° F for three hours. These granules were then broken down to No. 40 size. The resulting granules were compressed into tablets weighing approximately 100 mg. each.

Colorimetric assay showed each tablet to contain 0.95% phenolsulfonphthalein sodium.

Preparation of Phenolsulfonphthalein Tablets Using Cholesterol as a Base

Cholesterol U. S. P.	100 Gm.
Phenolsulfonphthalein sodium	1.00 Gm.
Alcohol	60 cc.

The cholesterol powder was granulated by using an alcoholic solution of phenolsulfonphthalein sodium. The wet mass was then passed through a No. 40 sieve and the resulting granules were dried at 120° F. The granules were then compressed into tablets weighing approximately 100 mg. each. These tablets asayed to be 0.75 % phenolsulfonphthalein.

Preparation of Phenolsulfonphthalein Tablets Using Pharmagel B as a Base

Pharmagel B	100 Gm.
Phenolsulfonphthalein sodium	1.0 Gm.
Alcohol	25 cc.

Pharmagel B granules were moistened with an alcoholic solution of phenolsulfonphthalein sodium and the wet mass was passed through a No. 20 sieve. The granules were dried at 120° F. and passed through a No. 40 sieve. These granules were compressed into tablets weighing about 100 mg. each. The tablets on assaying showed 0.55 per cent phenolsulfonphthalein.

TABLE II.—EXCRETION OF PHENOLSULFONPHTHALEIN AND DYE IN URINE OF RATS

Rat No.	Wt., Gm.	Wt. of Tablet, mg.	Amt. of Dye in Tablet, mg.	Per Cent of Phenolsulfonphthalein Excreted in Urine: 6 Hr.	12 Hr.	18 Hr.	24 Hr.	30 Hr.
				Carbowax®				
1	150	101.5	0.9135	95.6	...	...	...	...
2	140	99.3	0.8937	95.6	...	...	...	...
3	145	110.2	0.9942	100.0	...	...	...	...
4	145	110.8	0.9972	100.2	...	...	...	...
5	142	100.2	0.9018	98.47	...	...	...	...
Average Excretion of Dye:				97.97				
				Methocel® Carbowax® (1:1)				
1	145	110.3	1.0478	51.82	81.01	99.98	...	...
2	145	116.8	1.1096	53.42	81.99	99.99	...	...
3	150	106.0	1.0070	55.40	84.45	99.99	...	...
4	150	108.0	1.0269	56.73	85.09	99.98	...	...
5	145	110.0	1.0450	57.23	82.88	99.98	...	...
Average Excretion of Dye:				54.92	83.08	99.98		
				Methocel® Carbowax® (2:1)				
1	125	95.0	0.9262	57.55	89.20	99.99	...	...
2	120	89.7	0.8745	59.87	83.94	99.98	...	...
3	135	113.0	1.1017	31.75	77.15	94.68	99.92	...
4	130	97.5	0.9506	59.33	87.33	99.93	...	...
5	130	94.0	0.9165	63.51	87.83	99.99	...	
Average Excretion of Dye:				54.40	85.09	98.91	99.92	
				Methocel® Carbowax® (3:1)				
1	135	120.1	1.1409	10.99	61.77	99.98	...	...
2	130	129.0	1.2255	33.48	80.35	93.29	99.98	...
3	130	125.0	1.1875	23.75	60.00	91.25	99.95	...
4	135	126.0	1.1970	35.78	83.32	94.59	99.98	...
5	130	115.0	1.0025	36.20	79.30	...[a]	99.98	...
Average Excretion of Dye:				28.04	72.95	94.77	99.97	
				Methocel®				
1	140	127.0	1.2112	...[a]	71.91	99.99	...	...
2	135	111.0	1.0545	14.86	73.02	83.83	93.30	99.99
3	148	135.7	1.2891	45.87	67.42	84.85	99.97	...
4	135	126.0	1.1970	43.10	67.95	89.98	...[a]	99.98
5	145	130.0	1.2350	53.92	81.14	94.22	99.97	...
Average Excretion of Dye:				39.43	72.28	90.57	97.74	99.98
				Pharmagel B				
1	135	112.0	0.6160	26.76	99.99	...	...	...
2	130	135.0	0.7425	71.05	99.99	...	...	...
3	135	113.0	0.6215	70.51	99.99	...	...	...
4	130	115.6	0.6358	63.63	100.00	...	...	...
5	135	117.5	0.6462	66.23	99.97	...	...	...
Average Excretion of Dye:				59.63	99.98			
				Cholesterol				
1	140	111.0	0.8325	...[a]	53.38	74.88	89.98	99.98
2	132	109.8	0.8235	...[a]	69.10	89.19	99.99	...
3	138	112.0	0.8400	12.09	70.96	83.05	99.98	...
4	140	115.6	0.8670	13.60	77.6	90.32	99.99	...
5	135	110.0	0.8250	...[a]	76.72	90.51	99.99	...
Average Excretion of Dye:				12.84	69.55	85.59	97.98	99.98
				Sterotex®				
1	142	105.5	0.9495	...	...	...	...	...
2	135	107.0	0.9630	...	...	...	...	...
3	140	107.0	0.9630	...	...	...	...	...
4	140	105.6	0.9504	...	...	...	...	...
5	135	107.9	0.9711	...	...	...	...	...
Average Excretion of Dye:				...	...	...	...	...

[a] Rat did not urinate.

Preparation of Phenolsulfonphthalein Tablets Using Sterotex® as a Base

Sterotex powder	100 Gm.
Phenolsulfonphthalein sodium	1.0 Gm.
Alcohol	100 cc.

The Sterotex® powder was formed into a wet mass for granulation by an alcoholic solution of phenolsulfonphthalein, then passed through a No. 20 sieve and the resulting granules were dried at 40° C. These were passed through a No. 40 sieve and compressed into approximately 100-mg. tablets.

Colorimetric determination of the pellets showed that they contained 0.90% of phenolsulfonphthalein.

Experimental Animal.—The experimental animal for this investigation was the albino rat. The rat was selected because it is inexpensive, requires little space, and is easily handled. In primary study, as well as in evaluation of the bases, the experimental animal was the female albino rat weighing from 120 to 160 Gm.

Bishop and Folley (7) in 1944 have shown that the subcutaneous absorption in rats is twice that in humans. They suggested the possible reason may be the high basal metabolism in the rats.

Implantation Technique.—Except for some minor additions, the implantation technique used with phenolsulfonphthalein tablets was the same as that used with blank tablets. In the former case, every implanted rat was put into an individual metabolic cage and the urine collected every six hours.

Site of Implantation.—The site of implantation selected was about $^1/_4$–$^1/_2$ inch posterior to the ventral neck portion. It was selected so that the rat could neither scratch with its front or hind legs nor with its mouth. As this site was a little behind the joint of the neck and body there was no possibility for the tablet to become extruded by the movement of the neck.

Preparation for Incision.—The rat was anesthetized to the third stage of ether. Then it was put flat on the dorsal side and the neck portion was shaved and cleaned with 70 per cent alcohol. At once about a 3–4 mm. cut was made with a sterilized pair of scissors. Then by means of a blunt sterile probe, a pocket was made on the right or left side of the cut. The previously weighed tablet was put in this pocket by means of sterile forceps. In none of the cases did body fluids ooze out during implantation. The cut was carefully stitched with 11-mm. suture slips and treated with merthiolate solution to prevent infection. To keep the body fluids from oozing out flexible collodion was applied over the cut. An improperly placed implant shows a tendency to extrude. The rat was then weighed and put in a metabolism cage. The urine was collected every six hours and assayed colorimetrically by use of the Cenco-Sheard photelometer

Colorimetric Determination of Urine.—The volume of urine collected in six hours measured from 1 to 5 cc. per rat. This volume was made alkaline with 2 cc. 0.1 *N* sodium hydroxide and then diluted to 100 cc. This solution was assayed colorimetrically by the Cenco-Sheard photelometer. The procedure of collecting and assaying of urine was continued until the urine was free of phenolsulfonphthalein.

RESULTS AND DISCUSSION

In order to evaluate the implant bases, a water-soluble phenolsulfonphthalein was incorporated in those bases and then compressed into tablets. This phenolsulfonphthalein sodium, also called phenol red, being neither stored nor decomposed in the body, was the ideal indicator to evaluate the bases.

The thickness of the tablets and the compression were kept constant. Difference in densities and cohesive properties of the different base materials varied and to keep the above experimental conditions constant the weight of the tablets of one base was kept different from that of the other.

The relative efficiency of the various implant bases listed in Table II was calculated on the basis of excretion of the dye from the urine. After complete excretion of the dye, the urine was checked for twelve more hours.

Sterotex® Base.—The data recorded in Table II show that this base retains the dye as there was no excretion of the dye during the period of the experiment. For this reason, plus the fact that the base (insoluble in body fluids) was not absorbed even after eight weeks, Sterotex® was considered unsatisfactory (Table I).

Cholesterol Base.—Cholesterol base had been tried by Parkes (3) in 1942, who found that a highly water-soluble substance was extracted from the tablet by the tissues even when the base itself was not absorbed in weighable amounts. This was confirmed by the results in Table II. The phenolsulfonphthalein dye, which is highly water-soluble, was excreted up to an average of 12.84% in the first six hours. Colorimetric determination of excreted dye for the next six hours averaged 56.71%. Thus this water-insoluble base released an average of 69.55% of the dye in total within twelve hours. In eighteen hours after implantation 85.59% phenolsulfonphthalein was excreted and reached 97.98% total excretion in twenty-four hours. The rest of the dye was excreted in thirty hours as shown in Table II.

Thus this base, almost insoluble in water and not absorbed even after eight weeks, released the dye within thirty hours of implanting.

Pharmagel B Base.—Being slowly soluble in water and nontoxic, pharmagel B was tried as an implant base. As seen in Table II, it was found that the base itself lasts seventy-two hours in the body but releases 59.63% of the water-soluble dye in the first six hours. In twelve hours 99.98% excretion of the dye was obtained.

Pharmagel B, then, since it is eventually absorbed in seventy-two hours and produces no fibrous encapsulation, is of much greater value than either Sterotex® or cholesterol.

Carbowax® 6000 Base.—Carbowax® 6000 being slowly soluble in water, when tried as implant base was completely absorbed within thirty-six to forty-eight hours. This base can be used where encapsulation hinders the absorption of an active ingredient; it can release completely an active water-soluble ingredient in six hours.

Carbowax®-Methocel® (*1:1*) Base.—A mixture of Carbowax® and Methocel® was tried in an effort to decrease the rate of absorption of the dye. This mixture showed that it took nine to ten days for complete absorption of the base itself as compared to forty-eight hours with pure Carbowax® 6000. It also showed that it can hold on to the water-soluble dye longer than pure Carbowax® base. It can be seen from Table II, that Carbowax®-Methocel® (1:1) releases an average of 54.92% dye in the first six hours, 83.08% in the next six hours, and that all the dye is excreted at the end of eighteen hours.

Carbowax®-Methocel® (*1:2*) Base.—This mixture contained more Methocel,® which again decreases the rate of excretion, although not to any greater degree than Carbowax®-Methocel® (1:1) base.

The excretion lasted twenty-four hours in only one of a group of five rats. In all other cases complete excretion was obtained within eighteen hours.

Carbowax®-Methocel® *(1:3)* **Base.**—Carbowax®-Methocel® (1:3) was most efficient in holding up the dye for the longest time. The release of the dye in the first six hours averaged 28.04%. In the next six hours the total release reached 72.95% and in the following six hours release was complete in one rat; other rats showed total excretion of 91.25 to 94.5% in an eighteen-hour period. In twenty-four hours the dye was completely released in all four rats.

Methocel® 4000 Base.—This base seemed to be little more efficient than the other bases tested in restricting the release of dye. In the first six hours the released dye averaged 39.43%. Within the next six hours the total excretion of dye amounted to 72.28%. In eighteen hours one rat excreted all the dye completely while the others excreted an average of 86.97%; the average excretion of the four rats was 90.57%. In twenty-four hours only three out of four rats urinated; two showed complete excretion while one rat showed 93.8% excretion. In thirty hours the rat that had not urinated also showed complete excretion.

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Tables for the Identification of N. F. X Crystalline Substances by the Microscopic-Crystallographic Method*

By ALBERT H. TILLSON and WILLIAM V. EISENBERG

Optical crystallographic data for 184 N. F. X crystalline substances are given and arranged in a determinative table for rapid identification of specific compounds.

THE MICROANALYTICAL Laboratory receives many drugs for examination calling for the identification of specific ingredients which lend themselves to analysis by optical-crystallographic methods. The authors have had occasion to apply these methods to the identification of the substances listed in this report. The original data for these substances are derived either from determinations made by the authors on known crystalline compounds or from data reported in the literature. Where available, original literature references are given.

The application of the polarizing microscope to drug identification problems has been previously described (5, 7, 14, 19, 20). Every transparent crystalline substance possesses a unique set of optical-crystallographic properties which can be determined within certain limits by means of the polarizing microscope. By far the great mass of chemical drug products are susceptible of positive identification by means of this instrument. The identification involves microscopic-crystallographic observations of optical phenomena and measurements of physical constants for the positive identification of crystalline compounds.

The use of this analytical method for the qualitative and semiquantitative analyses of drugs merits greater attention than it has received heretofore, primarily because of its superiority in certain respects to other methods of analysis. It is of general value in the examination of all

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TABLE I.—OPTICAL CRYSTALLOGRAPHIC CONSTANTS OF SOME NATIONAL FORMULARY X CRYSTALLINE SUBSTANCES

Substance	System	N_α	$N\beta$ or N_i	N_γ	Optic Sign	2V	Extinction	Elongation	Habit
Acetanilid (19)	Rhombic	1.515	1.620	>1.733	+	88°	*P*	−	Thin plates
Acetarsone (Stovarsol)	Rhombic	1.495	1.714	1.770	..	...	*P*	−	Tiny rods
Acetophenetidin (19)	Monoclinic	1.518	1.574	>1.733	+	62°14′	..	..	.....
Acetylsalicylic Acid (15)	Monoclinic	1.505	1.645	1.655	−	Small	*P*	+	Lath-like
Allylbarbituric Acid (Sandoptal) (16-b)	Monoclinic	1.511	1.574	1.584	−	70°	*P*	+	Rods and plates
Ammonium Alum (38)	Isometric	1.459	...	...	..	...	..	..	Octahedral
Potassium Alum (38)	Isometric	1.456	...	...	..	...	..	..	Octahedral or cubic
Aminoacetic Acid (19)	Monoclinic	1.495	1.615	1.650	−	62°	..	..	Equant or brachydomatic
Aminopyrine (19)	?	1.520	...	1.732	..	...	..	..	.....
Ammonium Bromide (20)	Isometric	1.712	...	...	..	...	..	..	Tetrahedral
Ammonium Chloride (38)	Isometric	1.643	...	...	..	...	..	..	Tetrahedral
Ammonium Iodide (33)	Isometric	1.703	...	...	..	...	..	..	Cubic
Ammonium Salicylate (20)	?	1.570	1.695 (n_i)	1.710	..	...	*P*, *i*	..	Rods
Amobarbital (16-a)	Monoclinic	1.467	1.533	1.560	−	65°	*P*	+	Rods
Antimony Potassium Tartrate (19)	Rhombic	1.620	1.636	1.638	..	...	..	..	.....
Antipyrine (9)	Monoclinic	1.570	1.694	1.732	−	54°20′	..	..	Tablets
Aprobarbital (Alurate)	Monoclinic	1.520	1.581	1.600	−	Moderate	*i*	..	Rods
Arecoline Hydrobromide (20)	?	1.555	1.590	1.655	+	73°	*P*	−	Prisms
Arsenic Trioxide (26)	Isometric	1.755	...	...	..	...	..	..	Octahedra
Atropine (19)	Rhombic	1.550	1.583	1.595	..	...	..	..	.....
Atropine Sulfate (1)	?	1.555	..	1 60	..	...	..	..	.....
Barbital (19)	?	1.445	1.548	1.580	−	Moderate	..	..	Stubby prisms
Barbital Sodium (19)	?	1.512	1.532	1.615	+	40°	*P*	+	.....
Benzoic Acid (39)	Monoclinic	1.582	1.612	1.698	+	64°	..	..	Tablets
Betanaphthol (19)	Monoclinic	1.520	...	1.733	..	...	..	..	.....
Boric Acid (19)	Triclinic	1.340	1.456	1.459	−	7°	*i*	..	.....
Bromisovalum (Bromural), Phase I (39)	Triclinic	1.526	1.57	1.586	−	68°	..	..	Lamellar
Bromisovalum (Bromural), Phase II (39)	Rhombic	1.519	1.583	1.599	−	50°	..	..	Acicular
Butallylonal (Pernoston)	?	1.524	1.577	1.603	−	Moderate	*P*	+	Tiny rods and blades rosette aggregates
Butethal (Neonal) (16-b)	Monoclinic	1.456	1.520	1 552	−	72°	*i*	..	Rods and needles
Butethamine Hydrochloride (Monocaine HCl)	?	1.518	1.606	1.781	+	Large	*i*	..	Rods and plates
Caffeine (19)	?	1.455	1.472	1.733	..	...	*P*	−	Needles
Calcium Carbonate (26)	Trigonal	1.486 (ϵ)	...	1.658 (ω)	−	...	*P*	..	Varied, often rhombohedral
Calcium Chloride ($CaCl_2$) (38)	?	1.600	1.606	1.613	+	Moderate	..	..	.....
Calcium Chloride, hydrated ($CaCl_2 \cdot 6H_2O$) (38)	Trigonal	1.393 (ϵ)	...	1.417 (ω)	−	...	..	..	Prismatic
Calcium Hypophosphite (20)	Monoclinic	1.543	...	1.578	..	...	..	..	.....
Calcium Lactate (19)	?	1.470	...	1.510	..	...	*P*	+	Lath-like
Calcium Levulinate	Rhombic	1.495	1.507	1.537	+	Moderate	*P*	+	Long 6-sided rods
Calcium Pantothenate (18)	?	1.487	1.505	1.525	..	...	*P*	+	Small rods
Carbromal (35)	?	1.520	1.533	1.601	+	...	..	..	.....
Cetyl Alcohol (33-a)	?	1.480 (ω)	...	1.528 (ϵ)	+	...	..	..	.....
Chloramine-T	?	1.497	1.563	1.585	−	Large	*i*	..	Rods and plates
Chloroazodin (Azochloramid)	?	ca 1.570	...	>1.735	..	...	*P*	−	Small rectangular rods
Chlorprophenpyridamine Maleate (Chlor-Trimeton) (12)	?	1.533	1.668 (n_i)	<1.734	..	...	..	..	Box-like prisms

TABLE I (*Continued*)

Substance	System	$N\alpha$	$N\beta$ or N_i	$N\gamma$	Optic Sign	$2V$	Extinction	Elongation	Habit
Cinchophen (20)	Rhombic	1.545	1.575 (n_s)	>1.734	+	Moderate	P	−	Needle-like rods
Citric Acid (1 H_2O) (9)	Rhombic	1.493	1.498	1.509	+	65°42′	..	..	.....
Citric Acid (partly dried)	?	1.495	...	1.551	..	...	..	..	.....
Cocaine (39)	Monoclinic	1.49	...	1.58	..	Very large	..	..	Tablets
Cocaine Hydrochloride (19)	Rhombic	1.570	1.596	1.618	−	...	..	..	Thin micaceous plates
Codeine (1 H_2O) (16)	Rhombic	1.543	1.636	1.684	−	53°	..	..	Columnar
Codeine Sulfate (19)	Rhombic	1.561	1.642	1.661	−	ca. 50°	P	−	Rods
Coumarin (39)	Rhombic	1.56	...	>1.66	+	...	..	..	Rectangular plates
Cupric Sulfate (29)	Triclinic	1.514	1.537	1.543	−	58°	i	..	Short prismatic
Cyclobarbital (Phanodorn) (3)	Monoclinic	1.515	1.546	1.621	+	69°	P, s	±	Acicular
Danthorn (Istizin) (29-a)	Tetragonal	1.787 (ω)	...	1.945 (ϵ)	+	...	..	..	Tablets
Dextrose (1 H_2O) (17)	Monoclinic	1.521	...	1.549	+	Large	P	−	Plates, tablets
Diallylbarbituric Acid (Dial) (16-b)	Monoclinic	1.518	1.557	1.610	−	85°	s	−	Plates and prisms
Diethylstilbestrol (31)	Rhombic	1.594	1.611	1.73	+	42°	P	−	Long rods
Ephedrine Hydrochloride (34)	Monoclinic	1.530	1.603	1.638	−	70°	P	−	Prisms and rods
Ephedrine Sulfate (34)	?	1.540	1.555	1.587	+	Large	P	..	Hexagonal plates
Estradiol (Ovocylin)	?	1.550	1.596	1.661	+	Large	..	−	Rod-like prisms
Estriol (Theelol)	Monoclinic	1.528	1.642 (n_i)	1.680	−	Large	P	−	Rods
Ethyl Aminobenzoate (Benzocaine)	?	1.538	1.567	>1.744	+	Small	P, i	−	Lath-like rods and plates
Ethyl Vanillin	?	1.485	...	>1.733	..	...	...	..	.....
Glutamic Acid Hydrochloride	Rhombic	1.546	1.559	1.583	+	71°	P	±	Rods and prisms
Gold Sodium Thiosulfate (40)	Monoclinic	1.617	1.679	1.734	−	Large	i	−	...
Inositol (2 H_2O) (39)	Monoclinic	1.522	1.526	1.564	+	34°	..	..	Plates
Iodoform (39)	Hexagonal	1.750 (ϵ)	...	1.800 (ω)	−	...	..	..	Hexagonal plates or prisms
Lactose (1 H_2O) (36)	Monoclinic	1.517	1.553	1.555	−	Small	..	..	Wedge-shaped
Lanatoside C(32)	Monoclinic	1.502	1.524	1.568	+	71°	..	..	Tabular
Lead Acetate	?	1.559	1.576	>1.690	..	...	..	..	.....
Lithium Bromide (40)	Isometric	1.784	...	...	..	...	...	..	Cubic
Magnesium Hydroxide	Trigonal	1.559 (ω)	...	1.580 (ϵ)	+	...	..	..	.....
Magnesium Oxide (8)	Isometric	1.738	...	...	..	...	..	..	Cubes, octagons
Magnesium Phosphate, Tribasic (40)	Monoclinic	1.510	1.520	1.543	+	71°	...	..	.....
Magnesium Sulfate (19)	Rhombic	1.433	1.455	1.461	−	51°	P	..	Rods
Mannitol	Rhombic	1.520	1.555	1.558	−	...	P	+	Rods
Mechlorethamine Hydrochloride	?	1.535	1.550	1.612	+	Small	i	±	Plates and rods
Mephenesin	?	1.549 (ω)	...	1.616 (ϵ)	+	...	P	−	Rods, apparently uniaxial
Mephobarbital (Mebaral) (3)	Monoclinic	1.594	1.610	1.651	+	65°	P	−	Hexagonal tablets
Mercuric Iodide, Red (40)	Tetragonal	2.455 (ϵ)	...	2.748 (ω)	−	...	..	..	.....
Mercurous Chloride, Mild	Tetragonal	1.973 (ω)	...	2.656 (ϵ)	+	...	..	..	Pyramids or basal plates
Mercury Bichloride (40)	Rhombic	1.725	1.859	1.965	−	85°	..	..	Tablets and prisms
Metaphenilene Hydrochloride (Diatrin HCl) (11)	?	1.604	1.675	1.733	−	...	..	..	Six-sided rods
Methenamine (19)	Isometric	1.590	...	...	..	...	..	..	.....
dl-Methionine	?	1.544	1.582	1.600	..	...	P	−	Thin rods and plates
Morphine Sulfate (19)	Rhombic	1.545	1.620	1.632	−	...	P	−	Small rods and needles
Naepaine Hydrochloride (Amylocaine HCl)	?	1.498	1.585	1.734	..	...	P	+	.....
Nicotinamide (18)	Monoclinic	1.485	1.733 (n_i)	>1.733	..	...	P,	..	Needles

TABLE I (*Continued*)

Substance	System	N_α	N_β or N_i	N_γ	Optic Sign	2V	Extinction	Elongation	Habit
Nicotinic Acid (18)	Monoclinic	1.428	1.733 (n_i)	>1.733	..	....	*P*	..	Rod-like, fibrous
Para-Aminobenzoic Acid	Monoclinic	1.523	....	1.733	..	....	..	..	Rods
Penicillin Potassium	?	1.550	....	1.603	−	....	*P*	+	Elongated rectangular plates
Penicillin Procaine	?	1.545	1.570	1.685	+	....	*P*, *i*	±	Rods and prisms
Penicillin Sodium (6)	Rhombic	1.550	1.609	1.620	−	45°	*P*	+	Rods or plates
Pentobarbital Sodium (19)	?	1.477	....	1.523	..	....	..	..	.....
Phenacaine Hydrochloride (19)	Monoclinic	1.518	1.603	1.740	+	ca. 75°	..	..	Rhombic plates or needles
Phenobarbital (19)	?	1.557	1.620	1.667	−	80°	*P*	−	Six-sided rods
Phenolphthalein (19)	Triclinic	1.635	....	1.673	..	....	..	..	.....
Phenothiazine (20) (30-a)	Rhombic	1.610	1.734 (n_i)	1.95	+	81°	..	..	.....
Phenyl Salicylate (Salol)	Rhombic	1.532	1.620	>1.745	+	Large	*P*	..	Tablets
Picrotoxin	Rhombic	1.520	1.552	1.565	−	Small	*P*	+	Rods and prisms
Potassium Bicarbonate (29)	Monoclinic	1.380	1.482	1.578	−	81°	..	..	Tablets
Potassium Bitartrate (39)	Rhombic	1.511	1.550	1.590	−	88°28′	..	..	.....
Potassium Bromide (33)	Isometric	1.559	....	....	−	....	....	..	Cubic
Potassium Carbonate, hydrate (granular)	Rhombic	1.448	1.480	1.557	+	Moderate	*P*	−	Rods and plates
Potassium Chlorate (38)	Monoclinic	1.408	1.517	1.523	−	28°15′	*i*	..	Tablets
Potassium Citrate	?	1.510	1.520	1.555	..	....	..	..	.....
Potassium Guaiacolsulfonate (20)	Monoclinic	1.516	1.552	1.627	−	67°20′	..	..	.....
Potassium Iodide (33)	Isometric	1.667	....	....	..	....	..	..	Cubic
Potassium Nitrate (38)	Rhombic	1.334	1.505	1.506	−	7°12′	*P*	..	.....
Potassium Sodium Tartrate (19)	Rhombic	1.492	1.493	1.496	+	72°	..	..	Prisms, short
Potassium Thiocyanate (40)	Monoclinic	1.532	1.660	1.730	−	68°	..	+	Prisms, long
Probarbital Sodium (Ipral Sodium)	?	1.532	....	1.629	..	....	*P*	+	Tiny rods, needles
Procaine Hydrochloride (19)	?	1.540	1.556	>1.690	+	....	..	..	Six-sided plates
Promethazine Hydrochloride (Phenergan HCl) (12)	?	1.617	1.691	1.733	−	....	*P*	+	Rods
Prophenpyridamine Maleate (Trimeton Maleate)	Rhombic	1.548	1.574	1.665	+	Small	*P*	+	Rods and plates
Pyridoxine Hydrochloride (39)	Triclinic	1.480	1.706	1.734	−	33°	*i*	..	Thick plates, rods
Pyrogalloi	Rhombic	1.492	....	1.712	−	Small	*P*	−	Rods
Quinine (20, 39)	Rhombic	1.620	1.625	1.630	..	....	*P*	−	Small needles
Quinine Bisulfate (19)	?	1.555	....	1.620	..	....	*P*	+	Rods
Quinine Hydrochloride (19)	?	1.585	1.615	1.656	+	70°	*P*	+	Rods or flakes
Quinine Phosphate (39)	?	1.575	1.595	1.655	+	Moderate	*P*	+	Rods
Racephedrine Hydrochloride (23)	Monoclinic	1.570	1.608	1.630	−	70°	..	..	.....
Resorcinol (9)	Rhombic	1.578	1.620	1.627	−	46°	*P*	..	Thick prisms
Riboflavin, Phase I (Vitamin B_2) (39)	Rhombic	1.492	1.651	1.97	+	82°	*P*	−	Prisms or rods
Riboflavin, Phase II (Vitamin B_2) (39)	Rhombic	1.76	1.78	1.86	+	50°	*P*	+	Prisms or rods
Rutin (21)	?	1.508	1.734	>1.734	..	....	*P*	−	Rods
Saccharin (19)	Monoclinic	1.535	1.690	>1.733	−	Moderate	..	..	Equant
Saccharin Sodium (19)	?	1.560	1.642	1.733	+	ca. 87°	..	..	.....
Salicylic Acid	?	1.502	1.550 (n_i)	1.78	..	....	*i*	..	Rods
Santonin (39)	Rhombic	1.589	1.592	1.639	+	....	..	..	Prisms
Scopolamine Hydrobromide	Rhombic	1.567	1.585	1.623	+	Moderate	*P*	..	Rods and prisms
Silver Nitrate (38)	Rhombic	1.729	1.744	1.788	+	62°	..	..	Thick tablets
Sodium Acetate (Trihydrate) (39)	Monoclinic	1.416	1.463	1.482	−	62°50′	..	..	Short prisms
Sodium Benzoate (19)	?	1.490	....	1.680	..	....	..	..	Prisms
Sodium Bicarbonate (26)	Monoclinic	1.380	1.500	1.586	−	75°	..	..	Tablets
Sodium Biphosphate (1 H_2O) (38)	Rhombic	1.456	1.485	1.487	−	29°	..	..	Short prisms
Sodium Biphosphate (2 H_2O) (38)	Rhombic	1.440	1.463	1.482	−	83°	..	..	Pyramidal

TABLE I (*Continued*)

Substance	System	N_α	$N\beta$ or N_i	N_γ	Optic Sign	2V	Extinction	Elongation	Habit
Sodium Borate (38)	Monoclinic	1.447	1.470	1.472	−	39°	..	..	Six-sided plates
Sodium Bromide (33)	Isometric	1.641	...	...	..	...	..	..	Cubic
Sodium Cacodylate	?	1.478	1.485	1.503	+	Small	*i, s*	..	Large prisms
Sodium Chloride (38)	Isometric	1.544	...	...	..	...	..	..	Cubic
Sodium Glycerophosphate (6 H_2O) (40)	Monoclinic	1.444	1.455	1.468	+	85°	..	..	Prisms
Sodium Glycerophosphate (5 H_2O) (40)	Monoclinic	1.474	1.489	1.499	−	79°	..	..	.....
Sodium Iodide (33)	Isometric	1.775	...	...	..	...	..	..	Cubic
Sodium Phosphate, Dibasic (7 H_2O) (38)	Monoclinic	1.441	1.442	1.453	+	40°	..	..	Thick plates
Sodium Salicylate (19)	?	1.421	1.445 (n_i)	1.678	+	32°	*P*	+	.....
Sodium Sulfate (26)	Monoclinic	1.394	1.396	1.398	−	76°	..	..	Columnar tablets
Sodium Sulfite, Exsiccated (38)	Hexagonal	1.515 (ϵ)	...	1.565 (ω)	−	...	*P*	..	Prisms and tablets
Sodium Thiocyanate (40)	Rhombic	1.545	1.625	1.695	−	82°	*P*	−	Prisms
Sodium Thiosulfate (38)	Monoclinic	1.489	1.508	1.536	+	80°	..	..	Prisms or laths
Sorbitol (2)	?	1.51	...	1.54	..	...	*P*	+	Needles
Strychnine (39)	Rhombic	1.61	1.68	1.74	−	Large	*P*	+	Equant or prisms
Strychnine Nitrate (30)	Monoclinic	1.610	1.624	1.675	+	54°	*P*	−	Fine needles
Strychnine Phosphate (24, 30)	Monoclinic	1.589	1.597	1.655	+	Moderate	*P*	±	Needles
Strychnine Sulfate (5 H_2O) (30)	Monoclinic	1.592	1.597	1.661	+	10°	*P*	−	Heminorphic needles
Sucrose (9)	Monoclinic	1.540	1.567	1.572	−	48°	..	..	Plates
Sulfacetamide (4)	Rhombic	1.559	1.564	1.727	+	21°	*s*	..	Columnar
Sulfadiazine[a] (4)	Monoclinic	1.596	1.675	1.830	+	76°	*P, i*	±	Lath-shaped
Sulfadiazine[a] (22)	?	1.615	1.663	>1.734	..	...	*P*	±	Rods
Sulfaguanidine (1 H_2O) (4)	Monoclinic	1.586	1.649	1.731	+	86°	*P, i*	±	Lath-shaped
Sulfamerazine[a] (4)	Rhombic	1.568	1.657	1.687	−	58°	*P*	±	Equant or tabular
Sulfamerazine[a] (22)	?	1.587	...	1.675	..	...	..	..	.....
Sulfanilamide, Phase B, anhydrous (predominates in comm. preps.) (37)	Monoclinic	1.555	1.672	1.850	+	Large	*P*	−	.....
Sulfapyridine (4)	Monoclinic	1.670	1.736	1.813	+	88°	*P, i*	±	Tabular to equant
Sulfapyridine Sodium (1 H_2O) (19)	Rhombic	1.590	...	1.700	..	...	*P*	−	Rods
Sulfathiazole,[a] Phase I (22)	?	1.674	1.685	>1.733	+	Small	..	..	Hexagonal prisms
Sulfathiazole, Phase II (4)	Monoclinic	1.598	1.741	1.780	−	52°	*P, i*	±	Lath-shaped
Sulfathiazole[a]	?	1.695	1.733 (n_s)	>1.733	..	...	..	..	Hexagonal plates
Sulfathiazole Sodium (1½ H_2O)	?	1.596	...	1.621	..	...	..	..	Prisms
Sulfur, Sublimed (38)	Rhombic	1.958	2.038	2.245	+	68°58′	..	..	Pyramidal
Talc (26)	Monoclinic	1.539	1.589	1.589	−	0-30°	..	..	Fibrous fragments
Tartaric Acid (39)	Monoclinic	1.495	1.536	1.605	+	77°	..	..	Equant or plates
Terpin Hydrate (9)	Rhombic	1.505	1.512	1.524	+	77°	*P*	−	Stubby prisms and rods
Theobromine (25)	Monoclinic	1.435	>1.74	>1.74	−	30°	..	..	Needles
Theophylline (19)	Monoclinic	1.447	1.695 (n_i)	>1.733	..	..	..	..	Rods
Thiamine Hydrochloride (Form II) (27)	Monoclinic	1.600	1.639	1.685	+	87°	*i*	..	Lath-shaped plates and tablets. (This form most common in commercial preps.)
Thiamine Hydrochloride (Form I) (27)	Rhombic	1.618	1.640	1.714	+	61°	..	..	Elongated skeletal plates and tablets
Thymol (9)	Trigonal	1.525 (ω)	...	1.609 (ϵ)	+	...	...	..	.....
Tuaminoheptane Sulfate (23)	?	1.458 (ω)	...	1.468 (ϵ)	+	...	..	..	Plates and fibrous flakes
Vanillin (28)	Rhombic	1.551	1.694	1.80	−	75°	*P*	−	Plates and rods
Zinc Chloride (40)	?	1.687 (ω)	...	1.713 (ϵ)	+	...	..	..	.....
Zinc Phenolsulfonate (8 H_2O)	?	1.480	1.551	1.625	..	...	..	..	.....
Zinc Sulfate (7 H_2O)	Rhombic	1.462	1.486	1.490	−	46°14′	..	..	Acicular

[a] In the case of sulfadiazine, sulfamerazine, and sulfathiazole two sets of data are given. The second set in each case represents intermediate data, which are quite commonly found in some commercial samples. (Probably hydrous forms.)

Abbreviations or symbols: N_i = intermediate index. 2V = axial angle. *P* = parallel. *i* = inclined. *s* = symmetrical. ϵ = extraordinary index. ω = ordinary index.

TABLE II.—DETERMINATIVE TABLE FOR N. F. X, CRYSTALLINE SUBSTANCES, ARRANGED ACCORDING TO ASCENDING VALUE OF THE LOWEST INDEX

α	β	γ	Substance
1.334	1.505	1.506	Potassium Nitrate
1.340	1.456	1.459	Boric Acid
1.380	1.482	1.578	Potassium Bicarbonate
1.380	1.500	1.586	Sodium Bicarbonate
1.393 (*e*)	...	1.417 (*ω*)	Calcium Chloride hydrated ($CaCl_2 \cdot 6H_2O$)
1.394	1.396	1.398	Sodium Sulfate
1.408	1.517	1.523	Potassium Chlorate
1.416	1.463	1.482	Sodium Acetate (Trihydrate)
1.421	1.445 (n_i)	1.678	Sodium Salicylate
1.428	1.773 (n_i)	>1.733	Nicotinic Acid
1.433	1.455	1.461	Magnesium Sulfate
1.435	>1.74	>1.74	Theobromine
1.440	1.463	1.482	Sodium Biphosphate (2 H_2O)
1.441	1.442	1.453	Sodium Phosphate, Dibasic (7 H_2O)
1.444	1.455	1.468	Sodium Glycerophosphate (6 H_2O)
1.445	1.548	1.580	Barbital
1.447	1.470	1.472	Sodium Borate
1.447	1.695 (n_i)	>1.733	Theophylline
1.448	1.480	1.557	Potassium Carbonate, hydrate (granular)
1.455	1.472	1.733	Caffeine
1.456	...	...	Potassium Alum
1.456	1.485	1.487	Sodium Biphosphate (1 H_2O)
1.456	1.520	1.552	Butethal (Neonal)
1.458 (*ω*)	...	1.468 (*e*)	Tuaminoheptane Sulfate
1.459	...	...	Ammonium Alum
1.462	1.486	1.490	Zinc Sulfate (7 H_2O)
1.467	1.533	1.560	Amobarbital
1.470	...	1.510	Calcium Lactate
1.474	1.489	1.499	Sodium Glycerophosphate (5 H_2O)
1.477	...	1.523	Pentobarbital Sodium
1.478	1.485	1.503	Sodium Cacodylate
1.480 (*ω*)	...	1.528 (*e*)	Cetyl Alcohol
1.480	1.551	1.625	Zinc Phenolsulfonate (8 H_2O)
1.480	1.706	1.734	Pyridoxine Hydrochloride
1.485	1.733 (n_i)	>1.733	Nicotinamide
1.485	...	>1.733	Ethyl Vanillin
1.486 (*e*)	...	1.658 (*ω*)	Calcium Carbonate
1.487	1.505	1.525	Calcium Pantothenate
1.489	1.508	1.536	Sodium Thiosulfate
1.49	...	1.58	Cocaine
1.490	...	1.680	Sodium Benzoate
1.492	1.493	1.496	Potassium Sodium Tartrate
1.492	1.651	1.97	Riboflavin, Phase I (Vitamin B_2)
1.492	...	1.712	Pyrogallol
1.493	1.498	1.509	Citric Acid (1 H_2O)
1.495	1.507	1.537	Calcium Levulinate
1.495	1.536	1.605	Tartaric Acid
1.495	...	1.551	Citric Acid (partly dried)
1.495	1.615	1.650	Aminoacetic Acid
1.495	1.714	1.770	Acetarsone (Stovarsol)
1.497	1.563	1.585	Chloramine-T
1.498	1.585	1.734	Naepaine Hydrochloride (Amylocaine HC
1.502	1.524	1.568	Lanatoside C
1.502	1.550 (n_i)	1.78	Salicylic Acid
1.505	1.512	1.524	Terpin Hydrate
1.505	1.645	1.655	Acetylsalicylic Acid
1.508	1.734	>1.734	Rutin
1.510	1.520	1.543	Magnesium Phosphate, Tribasic
1.510	1.520	1.555	Potassium Citrate
1.51	...	1.54	Sorbitol
1.511	1.550	1.590	Potassium Bitartrate
1.511	1.574	1.584	Allybarbituric Acid (Sandoptal)
1.512	1.532	1.615	Barbital Sodium
1.514	1.537	1.543	Cupric Sulfate
1.515	1.546	1.621	Cyclobarbital (Phanodorn)
1.515 (*e*)	...	1.565 (*ω*)	Sodium Sulfite, Exsiccated
1.515	1.620	>1.733	Acetanilid
1.516	1.552	1.627	Potassium Guaiacolsulfonate
1.517	1.553	1.555	Lactose (1 H_2O)
1.518	1.567	1.610	Diallylbarbituric Acid (Dial)
1.518	1.574	>1.733	Acetophenetidin
1.518	1.603	1.740	Phenacaine Hydrochloride
1.518	1.606	>1.781	Butethamine Hydrochloride (Monocaine HCl)
1.519	1.583	1.599	Bromisovalum (Bromural), Phase II
1.520	1.533	1.601	Carbromal
1.520	1.552	1.565	Picrotoxin
1.520	1.555	1.558	Mannitol
1.520	1.581	1.600	Aprobarbital (Alurate)
1.520	...	1.732	Aminopyrine
1.520	...	1.733	Betanaphthol
1.521	...	1.549	Dextrose (1 H_2O)
1.522	1.526	1.564	Inositol (2 H_2O)
1.523	...	1.733	Para-Aminobenzoic Acid
1.524	1.577	1.603	Butallylonal (Pernoston)
1.525 (*ω*)	...	1.609 (*e*)	Thymol
1.526	1.57	1.586	Bromisovalum (Bromural), Phase I
1.528	1.642 (*n*	1.680	Estriol (Theelol)
1.530	1.603	1.638	Ephedrine Hydrochloride
1.532	1.620	>1.745	Phenyl Salicylate (Salol)
1.532	...	1.629	Probarbital Sodium (Ipral Sodium)
1.532	1.660	1.730	Potassium Thiocyanate
1.533	1.668 (n_i)	<1.734	Chlorprophenpyridamine Maleate (Chlor-Trimeton)
1.535	1.550	1.612	Mechlorethamine Hydrochloride
1.535	1.690	>1.733	Saccharin
1.538	1.567	>1.744	Ethyl Aminobenzoate (Benzocaine)
1.539	1.589	1.589	Talc
1.540	1.556	>1.690	Procaine Hydrochloride
1.540	1.565	1.587	Ephedrine Sulfate

Abbreviations or symbols: $2V$ = axial angle. P = parallel. i = inclined. s = symmetrical. e = extraordinary index. ω = ordinary index. N_i = intermediate index.

TABLE II (*Continued*)

α	β	γ	Compounds
1.540	1.567	1.572	Sucrose
1.543	...	1.578	Calcium Hypophosphite
1.543	1.636	1.684	Codeine (1 H_2O)
1.544	...	...	Sodium Chloride
1.544	1.582	1.600	*dl*-Methionine
1.545	1.570	1.695	Penicillin Procaine
1.545	1.575 (n_i)	>1.73	Cinchophen
1.545	1.620	1.632	Morphine Sulfate
1.545	1.625	1.695	Sodium Thiocyanate
1.546	1.559	1.583	Glutamic Acid Hydrochloride
1.548	1.574	1.665	Prophenpyridamine Maleate (Trimeton Maleate)
1.549 (ω)	...	1.616 (ε)	Mephenesin
1.550	1.583	1.595	Atropine
1.550	1.596	1.661	Estradiol (Ovocylin)
1.550	...	1.603	Penicillin Potassium
1.550	1.609	1.620	Penicillin Sodium
1.551	1.694	1.80	Vanillin
1.555	1.590	1.655	Arecoline Hydrobromide
1.555	...	1.60	Atropine Sulfate
1.555	...	1.620	Quinine Bisulfate
1.555	1.672	1.850	Sulfanilamide, Phase B, anhydrous
1.557	1.620	1.667	Phenobarbital
1.559	...	...	Potassium Bromide
1.559	1.564	1.727	Sulfacetamide
1.559	1.576	>1.690	Lead Acetate
1.559 (ω)	...	1.580 (ε)	Magnesium Hydroxide
1.560	1.642	1.733	Saccharin Sodium
1.56	...	>1.66	Coumarin
1.561	1.642	1.661	Codeine Sulfate
1.567	1.585	1.623	Scopolamine Hydrobromide
1.568	1.657	1.687	Sulfamerazine[a]
1.570	1.596	1.618	Cocaine Hydrochloride
1.570	1.608	1.630	Racephedrine Hydrochloride
1.570	1.694	1.732	Antipyrine
1.570	1.695 (n_i)	1.710	Ammonium Salicylate
ca. 1.570	...	>1.735	Chloroazodin (Azochloramid)
1.575	1.595	1.655	Quinine Phosphate
1.578	1.620	1.627	Resorcinol
1.582	1.612	1.698	Benzoic Acid
1.585	1.615	1.656	Quinine Hydrochloride
1.586	1.649	1.731	Sulfaguanidine (1 H_2O)
1.587	...	1.675	Sulfamerazine[a]
1.589	1.592	1.639	Santonin
1.589	1.597	1.655	Strychnine Phosphate
1.590	...	...	Methenamine
1.590	...	1.700	Sulfapyridine Sodium (1 H_2O)
1.592	1.597	1.661	Strychnine Sulfate (5 H_2O)
1.594	1.610	1.651	Mephobarbital (Mebaral)
1.594	1.611	1.73	Diethylstilbestrol
1.596	...	1.621	Sulfathiazole Sodium (1½ H_2O)
1.596	1.675	1.830	Sulfadiazine[a]
1.598	1.741	1.780	Sulfathiazole, Phase II
1.600	1.605	1.613	Calcium Chloride ($CaCl_2$)
1.600	1.639	1.685	Thiamine Hydrochloride (Form II)
1.604	1.675	1.733	Methaphenilene Hydrochloride (Diatrin HCl)
1.610	1.624	1.675	Strychnine Nitrate
1.61	1.68	1.74	Strychnine
1.610	1.734 (n_i)	1.95	Phenothiazine
1.615	1.663	>1.734	Sulfadiazine[a]
1.617	1.679	1.734	Gold Sodium Thiosulfate
1.617	1.691	1.733	Promethazine Hydrochloride (Phenergan HCl)
1.618	1.640	1.714	Thiamine Hydrochloride (Form I)
1.620	1.625	1.630	Quinine
1.620	1.636	1.638	Antimony Potassium Tartrate
1.635	...	1.673	Phenolphthalein
1.641	...	...	Sodium Bromide
1.643	...	...	Ammonium Chloride
1.667	...	...	Potassium Iodide
1.670	1.736	1.813	Sulfapyridine
1.674	1.685	>1.733	Sulfathiazole,[a] Phase I
1.687 (ω)	...	1.713 (ε)	Zinc Chloride
1.695	1.733 (n_i)	>1.733	Sulfathiazole[a]
1.703	...	...	Ammonium Iodide
1.712	...	...	Ammonium Bromide
1.725	1.859	1.965	Mercury Bichloride
1.729	1.744	1.788	Silver Nitrate
1.738	...	...	Magnesium Oxide
1.750 (ε)	...	1.800 (ω)	Iodoform
1.755	...	...	Arsenic Trioxide
1.76	1.78	1.86	Riboflavin, Phase II (Vitamin B_2)
1.775	...	...	Sodium Iodide
1.784	...	...	Lithium Bromide
1.787 (ω)	...	1.945 (ε)	Danthron (Istizin)
1.958	2.038	2.245	Sulfur, Sublimed
1.973 (ω)	...	2.656 (ε)	Mercurous Chloride, Mild
2.455 (ε)	...	2.748 (ω)	Mercuric Iodide, Red

[a] In the case of sulfadiazine, sulfamerazine, and sulfathiazole two sets of data are given. The second set in each case represents intermediate data, which are quite commonly found in some commercial samples. (Probably hydrous forms.)

Abbreviations or symbols: N_i = intermediate index. $2V$ = axial angle. P = parallel. i = inclined. s = symmetrical. ε = extraordinary index. ω = ordinary index.

solid materials. Direct microscopical examination reveals to the analyst at once a number of clues to the character of a sample, such as (1) the homogeneity of the material, (2) particle size and outward form, (3) the number of diverse ingredients in a mixture such as the kind and

number of excipients and active ingredients in tablets or capsules, and (4) impurities that may be present in a sample. In dealing with mixtures of chemical compounds, this method can readily identify the individual ingredients whereas the chemical analysis frequently affords only identification of the ions or chemical groups present. Ingredients often present in too small an amount to be handled by chemical methods can be readily identified by their optical crystallographic properties.

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A Double-Coloration Test for the Differentiation of Opium Alkaloids

By SHU-SING CHENG*

The discovery of a double-coloration test by combining Marquis reagent and Mandelin's reagent for the differentiation of opium alkaloids is reported.

THE DIFFERENTIATION of opium alkaloids in routine work depends chiefly on the colors that develop toward many dehydrating and oxidizing agents. Since the color produced from opium alkaloids is characteristic and the operation is simple and rapid, the color reactions have been widely used for identifying these alkaloids.

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In carrying out the identification work, several reagents should be used, since generally a certain reagent is only specific for a certain alkaloid, and it is rarely applicable to several alkaloids. In the case of a mixture, the reagents frequently give doubtful results. In this paper, a combination of reagents suitable for differentiation purposes is presented.

EXPERIMENTAL

Reagents.—*Marquis Reagent*—Prepared freshly by mixing 2 to 3 drops of 40% formaldehyde solution with 3 cc. of pure concentrated sulfuric acid.

Mandelin's Reagent.—Dissolve 1 Gm. of finely ground ammonium vanadate cold in 200 Gm. of pure concentrated sulfuric acid.

Procedure.—*For Dry Alkaloids.*—Place several minute crystals or a minute fragment of solid alkaloid on a white porcelain plate or in a white porcelain evaporating dish, add one drop of Marquis reagent and follow with one drop of Mandelin's reagent immediately.

For Alkaloidal Salt in Solution.—The test may be carried out either as free alkaloid by alkalizing the solution and extracting the base with organic solvent and drying, or as salt by directly drying the solution on a steam bath. In the former case, pour the solution into a separating funnel, add sufficient amount of ammonia test solution to make it alkaline and extract with alcohol-chloroform mixture (1:10), drain out several drops of the extract into a small porcelain dish, evaporate the solvent on a steam bath and dry for one minute, cool down, and add one drop of Marquis reagent followed with one drop of Mandelin's reagent.

If a test to be carried on a salt is desirable, simply dry the aqueous salt solution in a porcelain dish on a steam bath and apply the test as described above. Under this condition the salts in the form of sulfates are most satisfactory. Solutions containing chloride, carbonate, oxalate, or strong reducing agents give interference.

TABLE I.—COLOR REACTIONS OF SEVERAL OPIUM ALKALOIDS AND ALKALOIDAL MIXTURES TOWARD THE COMBINED REAGENT

Codeine	dull blue, green, dirty blue-violet
Morphine	peach-red, blue-violet, purple
Narcotine	orange, rose-red, cinnabar-red
Papaverine	brilliant greenish blue, beautiful sky-blue, which then fades to yellow
Thebaine	red-brown
Alkaloidal mixtures:	
Codeine and narcotine	blue mixed with cinnabar-red, steel-blue, blue-violet
Narcotine and morphine	peach-red mixed with cinnabar-red, violet mixed with green, purple
Narcotine and papaverine	greenish blue, blue, cinnabar-red
Papaverine and morphine	violet mixed with peach-red, bluish green, dirty purple

In the case of chloridec, hydrogen chloride gas is evolved upon addition of the reagent and foaming obscures the color. Carbon dioxide from carbonates is also troublesome. If any one of these constituents is present in the salt solution, it is necessary to convert the salt into the base, extract the alkaloid with organic solvent, and test as the dry base.

DISCUSSION

From the comparison of the colors developed toward several reagents, it has been found that even for five of the frequently encountered opium alkaloids, no single one of the well-defined reagents is available for universal differentiation among the alkaloids. The combination of Marquis reagent and Mandelin's reagent may be regarded as a suitable one for this purpose.

For the identification of papaverine, Warren (1) suggested a reagent by combining solid potassium permanganate and Marquis reagent. Papaverine gives a green color, almost instantly changing to blue and then bluish-green. In carrying out this test, the quantity of potassium permanganate crystals may influence the sensitivity of the test. If too much potassium permanganate is used, the color of potassium permanganate itself often disguises the expected coloration. Furthermore, the dryness of the potassium permanganate is essential for the success of the test; if the crystal is wet due to moisture adsorption, the test often fails. According to Autenrieth (2), of 39 alkaloids tested, the only one in any way simulating papaverine was an unnamed alkaloid separated from sanguinaria. The author, however, has found that codeine also shows a blue coloration with potassium permanganate and Marquis reagent, which confuses the color shown by papaverine so that the comparison with a known sample is frequently required. These drawbacks make Warren's test inconvenient. If the combination of Marquis reagent and Mandelin's reagent is used, these disadvantages are eliminated, since Marquis reagent shows the characteristic coloration of codeine first. In case of papaverine, it shows a red tint of little value in differentiation. The subsequent color shown by papaverine after the Mandelin's reagent is added is vivid greenish-blue and then beautiful sky-blue which persists for more than forty minutes and then fades to yellow. For codeine, the first color is that shown by Marquis reagent, namely, violet; when Mandelin's reagent is added, a dull blue color develops and then fades to dirty brown.

The double coloration is the advantage of the combined reagent and is important in distinguishing

TABLE II.—COMPARISON OF THE COLORS OF SEVERAL OPIUM ALKALOIDS TOWARD SEVERAL COLOR REAGENTS

Alkaloids	Mandelin's Reagent	Marquis Reagent	Fröhde's Reagent	Mecke's Reagent
Codeine	Slightly purple	Reddish, blue-violet, violet-blue	Violet-blue then green	Blue, quickly to emerald-green
Morphine	Slightly violet	Peach-red, violet-red	Violet, green	Blue, blue-green to olive-green
Narcotine	Cinnabar-red, crimson-red	Slightly purple then yellowish	Green, deep green, then fades	Greenish, blue, purple then cherry-red
Papaverine	Blue then green	Pink, reddish	Slightly violet then bluish	Greenish, dark steel-blue
Thebaine	Orange red, orange	Red-brown	Red-brown	Red-brown

alkaloids such as codeine from papaverine, codeine from morphine, papaverine from morphine, papaverine from narcotine. Otherwise several reagents specific for one of them would have to be employed. In the case of a mixture, the double-coloration test is especially useful in indicating of the presence or absence of a certain alkaloid. Under these circumstances, the single-coloration test often fails in predicating the presence of a certain alkaloid in an alkaloidal mixture, e. g., Marquis reagent fails to reveal papaverine in the presence of morphine, Mandelin's reagent fails to reveal papaverine in the presence of narcotine, and Mandelin's reagent fails to reveal codeine and morphine, respectively, in the presence of narcotine. Even in a pure state, it is difficult with Marquis reagent to differentiate codeine from morphine, especially when too much codeine is present. The double-coloration test by the combination of Marquis reagent and Mandelin's reagent is therefore useful in differentiating opium alkaloids especially for mixtures composed of two bases. The single reagent specific for a certain alkaloid often fails in these cases.

SUMMARY

A double-coloration test by combining Marquis reagent and Mandelin's reagent for the differentiation of opium alkaloids is suggested, and the discussion about its advantages is given.

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A Note on the Detection of Atropine Sulfate in the Presence of Butacaine Sulfate*

By W. EDWARD O'MALLEY† JANE W. FORREST,‡ and JOHN C. KRANTZ, Jr.§

ATROPINE SULFATE and Butacaine sulfate are frequently prescribed together in solution or in ophthalmic ointments. Difficulty has been encountered in attempting to establish the presence or absence of atropine in the presence of Butacaine Sulfate, U. S. P. We have obtained poor results in employing the tests set forth in U. S. P. XIII (1). Butacaine as well as atropine is capable of yielding a positive reaction. Beckurt's test (2), Eboli's test (3), and Ekkert's test (4) also fail to differentiate between the two compounds.

The two drugs may be differentiated by the Munch cat's eye test. Atropine produces mydriasis, and Butacaine does not. A simple qualitative chemical test appeared desirable.

We were able to detect atropine in the presence of Butacaine by warming a mixture of the two drugs with concentrated sulfuric acid. A pleasant odor resembling that of oil of rose was evolved. This is caused by the conversion of tropic acid into phenylethanol (5). Upon the addition of potassium dichromate the odor changed to that of bitter almond (5) owing to the oxidation of phenylethanol to benzaldehyde. Butacaine fails to give this reaction since it does not contain tropic acid. This reaction was reported by Rosenthaler in 1914 (6) and Hager in 1925 (7).

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Butacaine sulfate is sold as Butyn Sulfate® by Abbott Laboratories, North Chicago, Ill.
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Book Notices

Biochemistry of Cancer. By JESSIE GREENSTEIN. 2nd revised ed. Academic Press, Inc., Publishers, New York, N. Y., 1954. xii + 653 pp. Illustr. 16.5 x 23 cm. Price $12.

The second edition retains the general outline of the first edition. Some chapters have been pruned and some expanded to include new developments in oncology, especially carcinogenesis and chemotherapy. As in the first edition, Dr. Greenstein does not claim to have covered the entire field of the biochemistry of cancer, while of necessity he has included material from contiguous fields. There are sections on the induction of tumors, attempts at

control of tumor induction and of tumor growth, and the properties of tumors, and in the last chapter he outlines the present status of the problem. References are given at the conclusion of each chapter and the volume is interspersed with photographs, tables, and structures. It should be a valuable adjunct to the cancer research biochemist.

Harrison's Principles of Internal Medicine. 2nd ed. The Blakiston Company, Inc., New York. 1954. vii + 1703 + 87 pp. 26.5 x 20 cm. Price $16.

Little need be said about this encyclopedic work in the field of internal medicine. Its first edition, published in 1950, quickly acquired a place as an indispensable standard work in the reference library, in the physician's office and on the student's desk. The second edition has retained the basic outline of the first one, but many of the chapters dealing with specific diseases have been entirely rewritten and brought up to date.

History of Indian Pharmacy. By G. P. SRIVASTAVA. Vol. I. 2nd ed. Pindars Ltd., Calcutta, 1954. xvi + 277 pp. 22.5 x 14 cm. Price $2.50.

This book is composed of a series of articles which were published in the journal, *Indian Pharmacist.* They cover a period of prehistory, through ancient and mediaeval times. "The heaven-born science" of medicine and healing is attributed to the gods in legendary thought as outlined in a chapter on mythology. The author leads the reader along a semihistorical path to Charaka, who, although shrouded with mythical associations, left a concrete work, the *Charaka Samhita,* which attests to his existence in the earlier part of the first millennium B.C.

Because of the continuing revival of interest in ancient drugs, many will enjoy this book. The author's main concern, however, is in demonstrating that India's civilization predates that of Greece, Persia, and Arabia and influenced and contributed to those countries rather than the reverse. In their cultures, credit is given to Indian medicine and medicaments but the author has the grace to remark that although the Hindus were rich donors they were equally poor acceptors.

Legal Medicine, Pathology and Toxicology. By THOMAS A. GONZALES, MORGAN VANCE, MILTON HELPERN and CHARLES J. UMBERGER. 2nd ed., Appleton-Century-Crofts, Inc., New York, 1954. xii + 1349 pp. 25 x 17 cm. Price $22.

The first part of this new edition of a well-known standard work treats of legal medicine in the narrower sense of the term. It addresses itself mainly to the pathologist. The second part, however, which discusses forensic toxicology offers much of interest to the pharmacist. It includes discussion of occurrences such as administration of drugs by mistake or as a result of therapeutic error. There is also a thorough presentation of analytical toxicology based on data and experimental work from the Microchemical-Physical Laboratory of the City of New York.

The dominant position held by this book in the United States since 1940 will be strengthened by the additions and improvements of this second edition.

Meredith's Hygiene. 5th ed. The Blakiston Company, Inc., New York 1954. xv + 906 pp. 24 x 16 cm. Price $6.

While this book is intended to serve as a college text, it is equally suitable for the general reader. It is distinguished by brevity and up-to-dateness, even though one could wish for the explanation of some medical terms (e. g., "Koplik spots" is used on p. 285 without any comment) with which the lay reader cannot be expected to be familar. The illustrations are numerous and good throughout, except for the poorly reproduced frontispiece. As pharmacists, we are particularly pleased with the proper appreciation afforded our profession ("nearly as old and quite as honored as the medical profession is the profession of pharmacy") and with the intelligent treatment of such subjects as nostrums, proprietary-medicines, and self-medication.

The Practice of Modern Perfumery. By PAUL JELLENEK. Translated and revised by A. J. Krajkeman. Interscience Publishers, Inc., New York (J. W. Arrowsmith Ltd., Bristol, England), 1954. x + 219 pp. 22 x 14.5 cm. Price $4.75.

Perfume has been the subject of many exhaustive studies and here, Dr. Jellenek has attempted to cull from them the essential elements necessary for the young perfumer in the laboratory and to retell as lucidly and briefly as possible the history and basic principles of smelling, compounding, matching, and creating perfumes, the use of perfumery and aromatics in cosmetics, soaps, and many toilet articles. Tables are presented of perfume complexes and behavior of aromatics.

In addition to the technical aspects of the creation and manufacture of perfurmery the author stresses the importance of the influence of odors on the human senses. In Part IV he discusses the psychological effects produced and contrasts scents of stimulating and antierogenic properties.

Interesting to the lay woman is the section on matching perfume to personality.

The Surgical Appliance Technician's Handbook. By HOWARD L. BOYLAND. Surgical Appliance Industries, Inc., Cincinnati, Ohio, 1954. 459 pp. Illustrated. 16.5 x 24 cm. Price $10.

This is a book written by a practitioner for practitioners. The author, medical director of a surgical appliances firm has aimed at advancing the background knowledge and technical ability of those who, for one reason or another, have to deal with such matters as determining the need for trusses or supports, suspensories or elastic hosiery, the selection of the proper type of appliance in individual cases, and the fitting of the chosen appliance. The why and wherefore is explained with the help of many specially prepared anatomical drawings.

The retail pharmacist will also be able to derive from perusing the chapters on "Sources of Business," "The Duties of a Surgical Appliance Technician, and "Physical Considerations for the Surgical Appliance Department or Store." As far as we know, this book is unique in its approach and will more than repay its purchase price to those who plan to have an appliances department, or want to make an existing one more effective.

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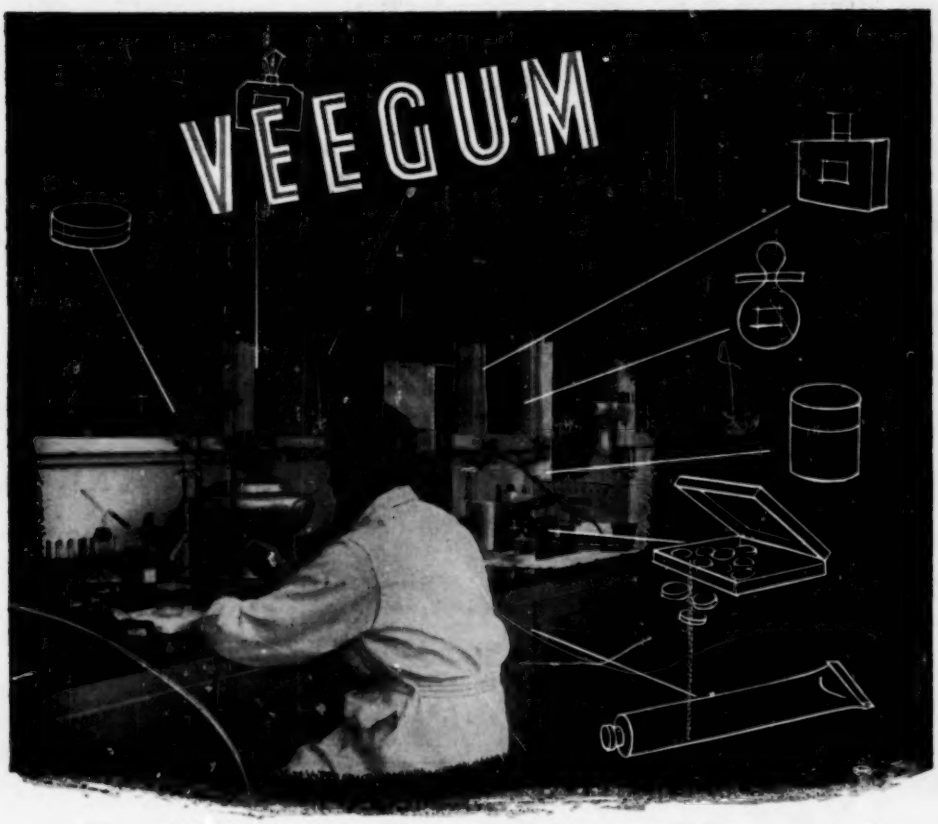

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